Altered intracellular calcium ion handling in airway smooth muscle and the pathogenesis of asthma

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Declaration

I hereby declare that the work contained within this thesis is my own. Technical assistance for myography, microscopy and some molecular biology techniques were kindly provided Dr Prieto-Lloret, Dr Snetkov and Dr Shaifta respectively.

Abstract

Asthma is characterised by airway inflammation, remodelling and bronchial hyperresponsiveness to normally innocuous stimuli. There is growing evidence that the airway smooth muscle (ASM) in asthmatic patients is altered and contributes to all three of these central pillars of the disease. Intracellular free calcium ions act as a secondary messenger within ASM in all of these processes and the expression of proteins regulating this tightly controlled process are altered in the disease. It has further been suggested that the long-term detrimental effect observed with some asthma therapies maybe associated with changes in calcium handling. The hypothesis of this study was that the calcium handling proteins SERCA2, TRPC3 and TRPC6 are altered by cytokines associated with asthma, or current therapies, leading to changes in calcium dynamics in the cell which contribute to the asthmatic phenotype. To investigate this hypothesis human primary healthy ASM cells were grown in culture and stimulated with either IL-13, TGF-β, TNF-α or a combination thereof or β_2-adrenergic receptor agonists. The RNA and protein expression of the aforementioned calcium regulators were measured by qPCR and western blot and the calcium dynamics assessed by loading cells with fura-2. The impact of reduced SERCA2 expression, as observed in ASM derived from asthmatics, was investigated in a murine model of ovalbumin induced allergic airway disease using $SERCA2^{+/-}$ mice. End-points included lung function measurements and inflammatory cell and cytokine infiltration. The key results found were that TGF-β differentially upregulates the expression of TRPC6 splice variants in a Smad2/3 dependent manner resulting in a reduction in flufenamic acid induced calcium entry. TNF-α upregulates TRPC3 expression while concomitantly down-regulating TRPC6 possibly conferring a switch in signalling from receptor operated calcium entry to store operated calcium entry. A reduction in SERCA2 in an *in vivo* model of asthma results in enhanced neutrophilia and an increase in sensitivity to methacholine to increase total airway resistance and decrease dynamic compliance. Finally it appears that chronic administration of β_2-adrenergic receptor agonists can lead to a variable decrease in SERCA2 expression however cAMP formation significantly increases it. Therefore β-arrestins may play a role in the detrimental effects observed with chronic dosing of this class of compounds. In conclusion the calcium handling proteins TRPC3 and TRPC6 are differentially altered by both TNF-α and TGF-β but unaffected by IL-13. The protein expression of SERCA2 on the other hand appears to be less regulated by the presence of asthmatic cytokines and its continued reduced expression in cultured asthmatic cells could be the result of genetic or epigenetic regulatory mechanisms.

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List of abbreviations

$[Ca^{2+}]_i$	Intracellular cytosolic calcium concentration
5-HT	5-hydroxytryptamine
5-HT_{2A}	5-hydroxytryptamine receptor 2A
5-LO	5-lipoxygenase
ACh	Acetylcholine
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
ASM	Airway smooth muscle
ATP(ase)	Adenosine triphosphate (triphosphatase)
AU	Arbitrary units
cADPR	Cyclic adenosine diphosphate ribose
CaMKII	Ca^{2+}/calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
CCE	Capacitative calcium entry
CD38	Cluster of differentiation 38
CICR	Calcium induced calcium release
COPD	Chronic obstructive pulmonary disease
cysLTs	Cysteinyl leukotrienes
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EGR1	Early Growth Reponse-1
ERK	Extracellular signal regulated kinase
FEV_1	Forced expiratory volume in one second
FEV_1	Forced expiratory volume in one second
GSK-3β	Glycogen synthase kinase-3β
GWAS	Genome-wide association studies
HDM	House dust mite
HEK293	Human embryonic kidney 293 cells
HLA	Human leukocyte antigen complex
IgE	Immunoglobulin E
IL	Interleukin
IL-1β	Interleukin-1 beta
IP_3	Inositol triphosphate
KC	Keratinocyte derived chemokine
kDa	Kilodalton
LABA	Long acting β_2-adrenergic receptor agonist
M_2AChR	Muscarinic acetylcholine receptor (subtype 2)
mAChR	Muscarinic acetylcholine receptor
MAPK `	Mitogen activated kinase
MBP	Major basic protein
MCU	Mitochondrial uniporter
MLC_{20}	Myosin light chain-20
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMLV	Moloney murine leukaemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MYPT1	Myosin phosphatase target subunit-1
NAADP	Nicotinic acid adenine dinucleotide phosphate
NCX	Sodium/calcium exchanger
NBF	Neutral buffered formalin
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
NHS	National Health Service
OAG	1-oleoyl-2-acetyl-sn-glycerol – DAG analogue
PDGF	Platelet derived growth factor
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1-α
PKA	Protein kinase A
PLC	Phospholipase C

PMCA	Plasma membrane calcium ATPase
PPAR-γ	Peroxisome proliferator-activated receptor γ
RANTES	Regulated upon activation, normal T-cells expressed and secreted
ROCE	Receptor operated calcium entry
ROCK	Rho kinase
RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic Ca^{2+} ATPase
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SOCE	Store operated calcium release
SP1	Specificity protein-1
SR	Sarcoplasmic reticulum
Stim1	Stromal interaction molecule-1
TFAM	Mitochondrial transcription factor A
TFB2M	Mitochondrial transcription factor B2
TGF-β	Transforming growth factor – beta
Th2	Type-2 T-helper cells
TNF-α	Tumor necrosis factor-α
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin-1
TSLP	Thymic stromal lymphopoietin
UPR	Unfolded protein response
β_2-AR	β_2-adrenergic receptor

Chapter 1 Introduction

Since Robert Boyle and Robert Hooke's experiments using a vacuum and a collection of unfortunate animals, described in the 1660 publication "New Experiments Physico-Mechanical: Touching the Spring of the Air and their Effects" it has been known that the presence of air is critical for respiration. A fact that is not lost on asthma sufferers worldwide today. Prior to that work, a direct link had not been established and it has since paved the way for hundreds of years of research aimed at increasing ventilation of the body.

1.1 Asthma

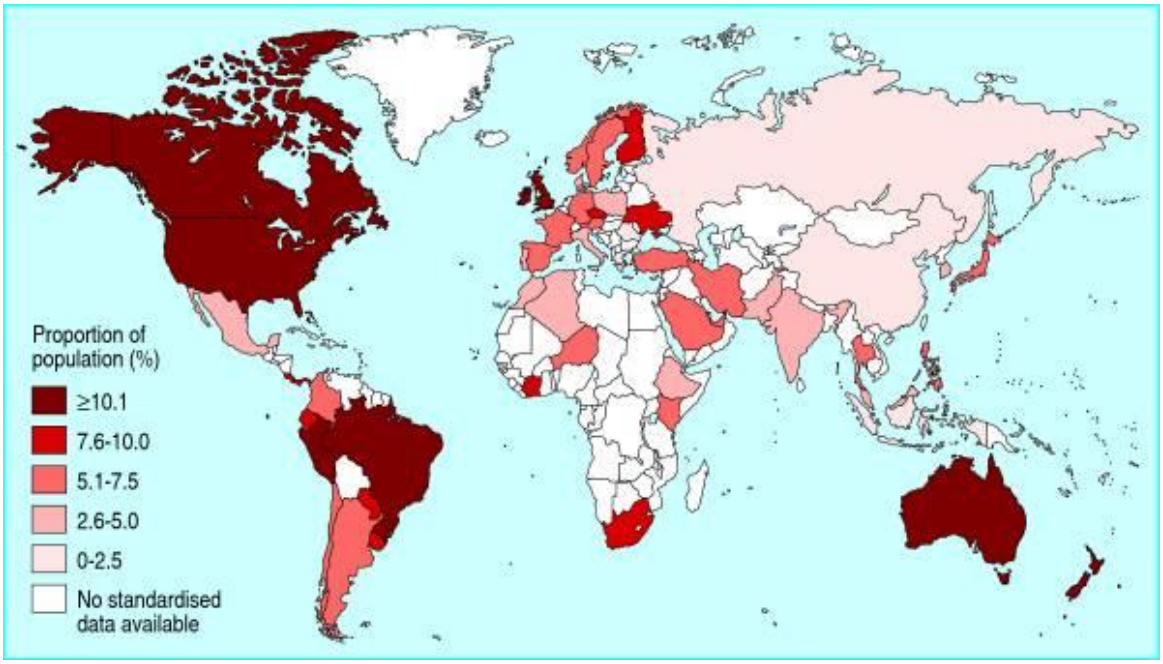
Asthma is a chronic inflammatory disease of the airways with over 5.4 million people in the United Kingdom receiving treatment for it in 2012 (NHS). The disease is characterised by airway remodelling, an enhanced inflammatory milieu in and around the airways, hypersensitivity and hypercontraction to allergens. The culmination of these effects results in impaired gaseous exchange between the pulmonary vasculature and the lung. The clinical manifestations of the disease arise as chest tightness, wheezing, cough and dyspnoea.

1.1.1 Burden and prevalence

The estimated annual cost of in-patient care, general practitioner consultations and prescriptions in the UK in a 2004 survey was £63.1M (Gupta *et al.*, 2004) and Asthma UK estimates the NHS spends around £1 billion a year in total on treating and caring for asthma patients.

Asthma is least common in Africa and the Far East and most common in economically developed countries which are more urbanised (Masoli *et al.*, 2004). The life-style that accompanies these countries favour the development of asthma as air quality is generally lower and common allergens such as house dust mites are more abundant. The "hygiene hypothesis" attempts to explain the causal relationships between early life pathogen exposure, close proximity to other children and other risk factors more commonly encountered in the developing world through demonstration of the inverse relationship between hay fever risk and family size (Strachan, 1989). Although many of these factors have been shown to be inversely correlated to asthma prevalence there are inconsistencies in the findings made (Ramsey *et al.*, 2005). For example it has been hypothesised that a T_H1 stimulus in the first two years of life can suppress the over-development of the T_H2 response which can lead to atopy (Arkwright *et al.*, 2001; Erb *et al.*, 1999). However, helminth exposure generating a T_H2 response can suppress an allergen specific response in the lung (Wang *et al.*, 2001). Clearly there are links between allergy risk in later life and early exposure

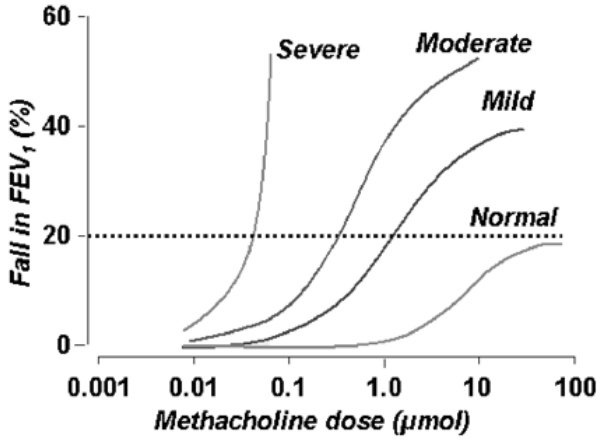
to various antigens with evidence coming from epidemiological studies, however the underlying molecular pathways involved are still to be fully described (Wills-Karp *et al.*, 2001).



1.1.1-1 Global prevalence of asthma. Asthma's prevalence is highest in urbanised "Western" countries and as more countries develop economically it is predicted there will be a further 100 million asthmatics globally by 2025 (Masoli *et al.*, 2004).

1.1.2 Definition and epidemiology

Although asthma is termed as one disease there are many variations of it with some overlapping and non-overlapping aspects between patients. Common to all asthmatics is a hypersensitivity to normally innocuous stimuli causing broncho-constriction and the extent of this correlates well with the severity of asthma (Holgate, 2004).



1.1.2-1 Typical dose response curves of patients suffering from severe asthma to the healthy population. The graph illustrates the bronchial hypersensitivity (left-ward shift) and hyperresponsiveness (increase in slope) present in asthmatics (Holgate, 2004).

Asthma has a complex pathophysiology which was classically categorised into two broad different subsets: extrinsic, where a definite external cause can be identified to which the body generates a specific IgE response (Martinez *et al.*, 2013); or intrinsic, where no such environmental stimuli exists (Turato *et al.*, 2008). These terms are also known as atopic and non-atopic asthma respectively. It is now becoming increasingly clear that more specific "endotyping" is essential to developing novel therapies for severe patients who are not controlled by β_2-adrenoceptor (β_2-AR) agonists or steroids (Anderson, 2008; Wenzel, 2013). Instead of basing research and diagnosis on set phenotypes such as propensity for exacerbations there has been a call to shift towards clearer biomarkers or genetic risk factors to define endotypes (Anderson, 2008).

1.1.3 Genetic influence

Many studies have shown that asthma runs in families, however not always in line with Mendelian inheritance patterns, suggesting both genomic and epigenetic heritable risk factors being passed on (Durham *et al.*, 2011). Genome-wide association studies (GWAS) have discovered genetic loci linked with asthma prevalence at or near the genes of SMAD3, IL-6R, IL-18 and ORMDL3 (Ferreira *et al.*, 2011; Moffatt *et al.*, 2010) (see section 1.6.2 for more on ORMDL3). In addition, the human leukocyte antigen complex (HLA) is involved in immunomodulation and has been linked with asthma (Nicolae *et al.*, 2005). It has been linked with maternal inheritance via allelic imprinting or other epigenetic mechanisms inducing bronchoconstriction (Durham *et al.*, 2011; Nicolae *et al.*, 2005), thus further adding to the complexity of inheritance of the disease amongst the population.

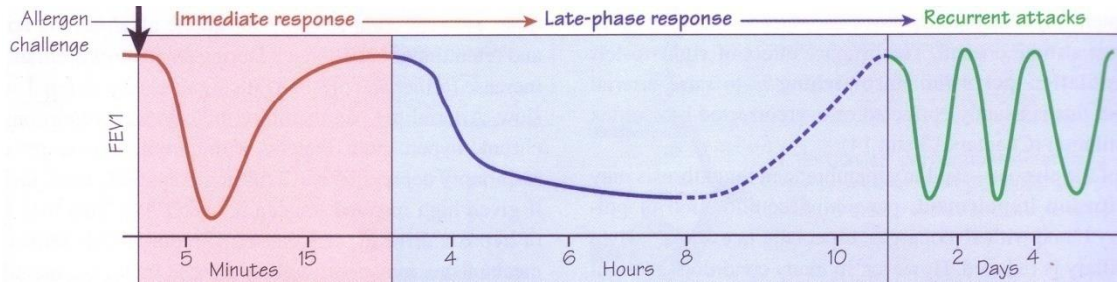
Another important genetic variation in asthma is the effect gender plays on its pathogenesis. It is well described that there is a higher incidence of asthma in boys than in girls, however around adolescence and puberty the pattern is reversed (Choi, 2011). It has been hypothesised that this occurs because of slower pulmonary development in males and also a potential role of sex hormones as those from males have been shown to suppress asthma whereas female sex hormones aggravate it (Osman, 2003). This hypothesis has been backed up in a murine model of asthma showing testosterone to have a protective effect in BALB/c mice displaying a reduced T_H2 response (Hayashi *et al.*, 2003). Female BALB/c mice have also been shown to experience more airway remodelling (discussed in more detail in section 1.4) than male mice, a factor of severe irreversible asthma (Takeda *et al.*, 2013). However, the picture is not that straight forward, as estradiol administration has been associated with improving asthma symptom scores in mild to moderate asthmatics with premenstrual asthma (Chandler *et al.*, 1997). Furthermore, the *in vivo* effects of androgens in mice appear to promote AHR by enhancing the vagally mediated reflex pathway affecting airway tone (Card *et al.*, 2007).

1.2 Inflammation and immunology

Asthma is commonly characterised in many patients by an elevation of eosinophils, goblet cell hyperplasia and CD4+ T-cells secreting the classical T_H2 cytokines IL-4, IL-5 and IL-13 and most recently regulatory T-cells (Treg) (Martinez *et al*., 2013). The innate immune system is also involved heavily, with the airway epithelium playing an important role limiting the early growth and expansion of microbes while antigen specific T-cell responses are established (Koyasu *et al.*, 2013). In response to an allergen the epithelium produces thymic stromal lymphopoietin (TSLP), IL-25 (IL-17E), IL-33 and CC family cytokines to activate CD4+ T-cells, mast cells, basophils, eosinophils and the newly discovered nuocytes (Locksley, 2010; Neill *et al.*, 2010; Smith, 2010). The nuocytes are capable of contributing to the inflammation by releasing IL-5 and IL-13 in response to IL-33 which could promote T_H2 differentiation leading to IgE production (Martinez *et al.*, 2013), eosinophilia and goblet cell hyperplasia (see 1.2.1 below). IL-33 acts as an alarmin and is localised in the nucleus of fibroblasts, epithelial and endothelial cells so that upon their necrosis it is released to stimulate a T_H2 response (Koyasu *et al.*, 2013).

Symptoms manifest themselves often in a biphasic manner termed the early and late phase response (see Fig. 1.1.3-1). The early phase is short lived, up to 20 minutes, and involves the degranulation of mast cells through IgE crosslinking FcεR1 releasing numerous mediators including histamine, prostaglandin D2 and leukotrienes (Turner *et al.*, 1999). The resulting impact of the degranulation is smooth muscle constriction (Dahlen *et al.*, 1980) and inflammatory cell migration (Spada *et al.*, 1994) see Fig. 1.2.2-1. The late phase

response occurs following a return to baseline of lung function several hours later and involves the adaptive immune response initiated though antigen presentation by dendritic cells leading to T-cell differentiation. The differentiated (T_H2) cells release IL-4, IL-13 and CD40L act on naïve B-cells to induce IgE class-switching (Akdis *et al.*, 2012). The cytokines released by T_H2 cells lead to further eosinophil and neutrophil recruitment into the airways which release a variety of mediators leading to airway remodelling, mucus secretion, oedema and bronchoconstriction (all discussed in more detail later) contributing to the late phase effect.



1.1.3-1 Typical response timeline of an atopic asthmatic to inhaled allergen, (Ward, Ward and Leach, 2010: 56). Here the early and late phase responses are termed "immediate" and "late-phase" response. The early phase is largely a result of mast cell degranulation due to IgE binding to surface receptors. The late phase on the other hand involves components of the adaptive immune response leading to eosinophil recruitment, mucus secretion and a prolonged decrease in FEV1.

1.2.1 IL-5 and eosinophilia

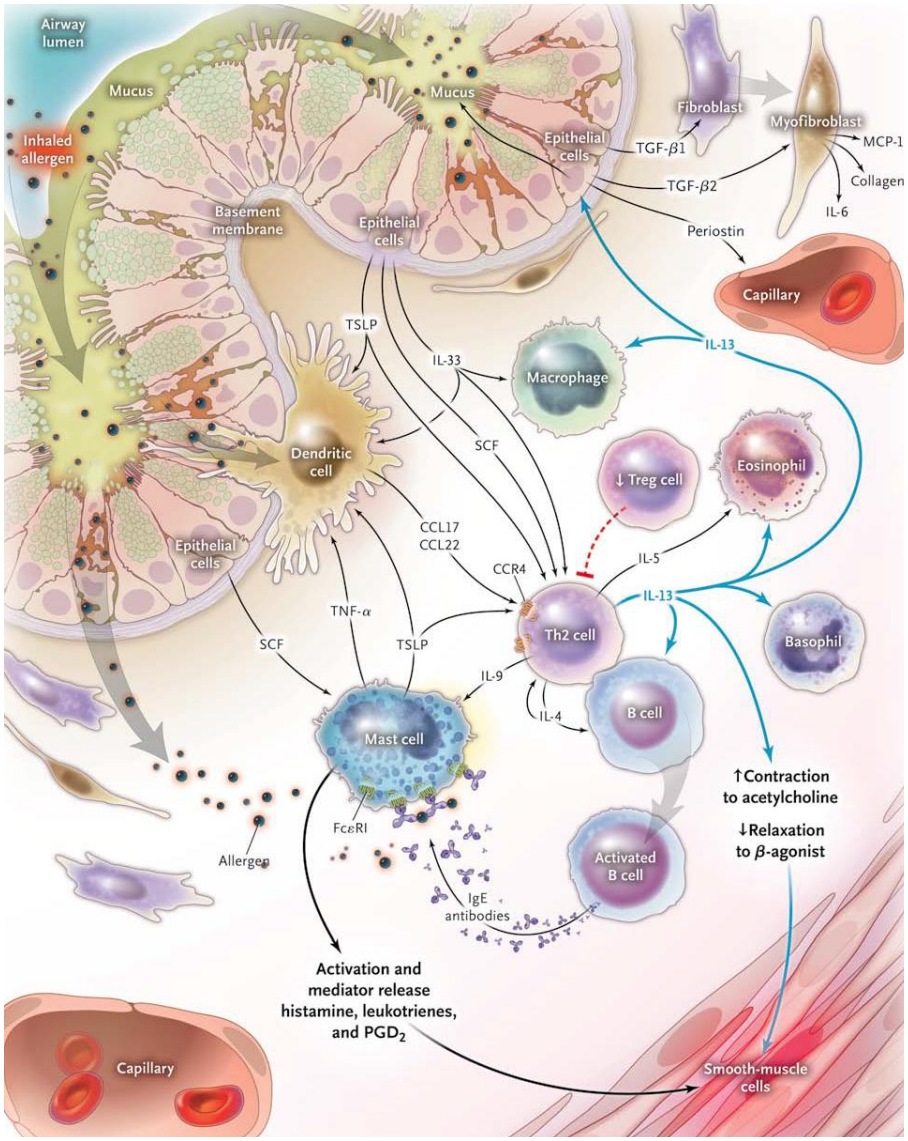
Asthma can be divided into eosinophilic and non-eosinophilic subtypes. The majority of exacerbations are non-eosinophilic and are not prevented by corticosteroid treatment (Jayaram *et al.*, 2006). However eosinophils are very much abundant in allergic asthma and IL-5 plays a key role in their differentiation and survival. Treatment with mepolizumab an anti-IL-5 monoclonal antibody has been shown to improve the quality of life in refractory eosinophilic asthmatics. This was achieved by reducing eosinophils in the blood and the sputum thus reducing the frequency of exacerbations (Haldar *et al.*, 2009). A double airway transgenic mouse expressing IL-5 systemically in T-cells and eotaxin-1 in the airway epithelium further highlight the role of eosinophils and IL-5 in asthma (Ochkur *et al.*, 2007). The mice displayed many characteristics of severe asthma in the absence of allergen such as airway remodelling and AHR. Eotaxin, an eosinophil chemokine has been shown to be released from asthmatic ASM cells between 2.5 and 6 fold more compared to "healthy" ASM cells after stimulation with a variety of pro-inflammatory cytokines usually present in asthma *in vitro* (Chan *et al.*, 2006).

Eosinophils can contribute to airway remodelling (see 1.4) by releasing cationic proteins such as eosinophil cationic protein (ECP), major basic protein (MBP) and TGF-β (Hisamatsu *et al.*, 1990; Wong *et al.*, 1991; Zagai *et al.*, 2007). A component of airway remodelling, ASM proliferation, has been shown to be increased by eosinophils isolated from mild asthmatics and not healthy controls (Halwani *et al.*, 2013). The authors discovered that the interaction was dependent upon cell-cell interactions between ASM and eosinophils resulting in their release of cysteinyl leukotrienes and not the release of ECM from the ASM. Prevention of eosinophil extravasation into the lung and subsequent adhesion onto ASM could be a useful therapeutic approach.

1.2.2 IL-13

IL-13 has been shown to play a central role in allergic asthma, directing many of the processes involved in the response. Upon exposure to an inhaled allergen, mast cells are activated and IL-13 drives IgE class switching in B-cells and that the class switching is more IL-13 dependent in allergic asthma patients compared to IL-4 (Van der Pouw Kraan *et al.*, 1998). T cells (van der Pouw Kraan *et al.*, 1996), basophils (Li *et al.*, 1996), mast cells (Pawankar *et al.*, 1997) and ASM (Grunstein *et al.*, 2002) are all sources of IL-13 in the airways. Once released it activates and prolongs the survival of eosinophils and is involved in their chemotaxis (Horie *et al.*, 1997).

Blockade of IL-13 using a soluble fusion protein in a murine model of asthma, post sensitisation but prior to ovalbumin challenge, reversed both AHR and mucus production in an eosinophilic and IgE independent manner (Wills-Karp *et al.*, 1998). The study also shows administration of recombinant IL-13 can induce pulmonary eosinophilia and AHR in mice, two key markers of asthma. At a cellular level IL-13 has been shown to alter calcium handling dynamics via an up-regulation of CD38 protein expression in ASM leading to enhanced mobilisation following agonist stimulation (Deshpande *et al.*, 2004). CD38 generates cADPR production which can directly or indirectly interact with the ryanodine receptor to increase the open probability of the channel (Jude *et al.*, 2008). Blockade of cADPR with 8-bromo-cADPR attenuated the IL-13 augmented calcium response showing that CD38 may play an important role in the IL-13 induced asthmatic phenotype. The enhanced mobilisation observed could contribute to airway remodelling and a worsening of symptoms (see section 1.4). An overview of the allergic response and airway inflammation is displayed in Fig. 1.2.2-1 (Kraft, 2011).



1.2.2-1 An overview of the complex inter-cellular interactions in asthma following inhalation of allergens. IL-13, TGF-β and TNF-α all play a role in the sensitisation of airway smooth muscle to agonists, enhancement of proliferation and the airway inflammatory response (Kraft, 2011).

1.2.3 IL-8 and neutrophils

The inflammation present in the airways and bronchioles of severe asthma differs from that of mild to moderate asthmatics in that neutrophils are the primary inflammatory cell not eosinophils (Wenzel *et al.*, 1997) and that they remain present even with corticosteroid treatment. Neutrophils release a variety of mediators which contribute to both the early and late phases of the asthmatic response. Matrix metalloproteinase-9 (MMP-9), elastase, reactive oxygen species, thromboxane A_2 and myeloperoxidase are all released in the early phase and cause cytotoxicity, mucus hypersecretion, bronchoconstriction on bronchial hypersensitivity (Monteseirin, 2009). It has been suggested that neutrophils add to the late phase response by secreting IL-8, (murine functional equivalent is KC) their most potent chemokine to promote

their own recruitment and activation (Monteseirin, 2009). ASM has been shown to be a source of IL-8 in culture following stimulation with TNF-α and IL-1β (John *et al.*, 1998). Originally just thought to be released by eosinophils, there is some evidence ECP is also released from neutrophils on a much slower time-scale adding to the late-phase cytotoxicity (Monteseirin, 2009). Many of both the early and late-phase neutrophil factors that alter ASM function stem from changes in intracellular calcium signalling (see 1.5).

ASM cells can secrete pro-inflammatory cytokines, chemokines and growth factors in both a paracrine and autocrine manner to aid leukocyte activation and adhesion and furthermore to enhance both the ASM contractile and proliferative responses (Hirst, 2003). T_H2 cytokine stimulation of asthmatic ASM cells induces mast cell migration into the muscle bundle possibly via IL-8 and eotaxin. Furthermore, supernatant from ASM derived from healthy patients inhibits migration to asthmatic derived ASM cells (Sutcliffe *et al.*, 2006).

1.2.4 TGF-β

The TGF-β superfamily consists of numerous related extracellular signalling molecules with widespread roles ranging from regulation of development (Morty *et al.*, 2009), modulation of the immune response (Tiemessen *et al.*, 2003) to proliferation (Cohen *et al.*, 1997). In humans TGF-β consists of three protein isoforms encoded by separate genes and expressed in a tissue specific manner, TGF-$\beta_{1\text{-}3}$. TGF-β_1 (henceforth abbreviated as TGF-β) is the most abundant isoform found in mammals and the prototype for this family.

TGF-β is synthesised as a larger precursor molecule composed of a pro-domain, the active molecule and a latency associated peptide. The pro-domain is cleaved but remains non-covalently associated with the active molecule after secretion where it is stored in the ECM in its inactive form. Cleaving of the covalent bond between the latency associated peptide and the active molecule by a number of different factors enables the TGF-β to bind to one of its receptors and initiate signalling. There are three related but functionally distinct serine-threonine kinase receptor proteins called TβRI, TβRII and TβRIII. TGF-β binds to the constitutively active TβRII which induces a heterotetrameric complex with TβRI, phosphorylating it in the process, ultimately leading to a cytoplasmic signalling cascade via Smad phosphorylation.

TGF-β plays a central role in the tissue remodelling process as it can cause fibrosis (Gordon *et al.*, 2008) and ASM proliferation (Oenema *et al.*, 2013b). Furthermore the ASM proliferation is mediated via ECM-

integrin interactions which can be enhanced by M_3AChR stimulation, making it all the more relevant in asthma. TGF-β also serves as a negative regulator of the inflammatory response and therefore its actions are essential in immune homeostasis (Haneda *et al.*, 1999; Kulkarni *et al.*, 1993). Levels of TGF-β have been shown to be elevated in the lungs of asthmatics (Redington *et al.*, 1997) and it is believed that this is part of a compensatory reaction to prevent excessive inflammation and AHR in response to allergen (Groneberg *et al.*, 2004). The tissue remodelling is a deleterious by-product leading to some of the irreversible effects of asthma due to its continued presence in the airways.

Smads are common intracellular mediators of TGF-β signal transduction. They can be divided into three sub-classes, receptor-activated Smads (R-Smads), common partner Smads (co-Smads) and inhibitory Smads (anti-Smads) (Piek *et al.*, 1999). Smad2 and Smad3 are mediators of the TGF-β signalling cascade (R-Smads) and are phosphorylated by TβRI (Macias-Silva *et al.*, 1996; Nakao *et al.*, 1997). Smad4 is a co-Smad and aids the efficient translocation of Smad2/3 to the nucleus for TGF-β signalling (Nakao *et al.*, 1997). As mentioned previously (see 1.1.3), a genome wide association study implicated a SNP in the Smad3 gene with increased asthma prevalence (Moffatt *et al.*, 2010). Smad2 has also been implicated in asthma as over-expression in airway epithelium in a house dust mite (HDM) murine model of the disease increased AHR and remodelling markers (Gregory *et al.*, 2010). The TGF-β Smad pathway has been shown to control the expression of certain proteins not only on a genomic level but also at an epigenetic level (Thillainadesan *et al.*, 2012). The paper outlines the dynamic nature of methylation and demethylation and what its role may be in the context of certain cancers. Abnormal signalling of TGF-β is very much present in asthma too and dysregulation of specific DNA methylation may play a critical role in this.

1.2.5 TNF-α

TNF-α has been shown to be greatly elevated in the airways in symptomatic asthmatic patients compared to non-symptomatic patients (Broide *et al.*, 1992). Many cells in the airway store TNF-α and have the ability to release it upon an antigen entering the lung including mast cells, eosinophils, epithelial cells and macrophages (Costa *et al.*, 1993; Gosset *et al.*, 1991; Khair *et al.*, 1994; Plaut *et al.*, 1989). It may then act on airway smooth muscle (ASM) to cause bronchoconstriction and infiltration of neutrophils (Thomas *et al.*, 1995). The bronchoconstriction may be a result of both calcium sensitisation (Parris *et al.*, 1999) or changes in calcium handling (Amrani *et al.*, 1997; Jia *et al.*, 2013; Sathish *et al.*, 2012; Sathish *et al.*, 2009). A comprehensive pharmacological profile has been performed in guinea pigs, both *in vivo* and *ex vivo* to discover targets of TNF-α in eliciting bronchoconstriction (Makwana *et al.*, 2012). Both 5-HT and

electrical field stimulation responses were enhanced by TNF-α however the methacholine response was unchanged, suggesting a pre-synaptic action on parasympathetic nerve terminals. The enhanced response to 5-HT was abolished in epithelium denuded trachea and with pre-treatment with ketanserin and atropine. A potential mechanism of action is suggested as enhanced 5-HT(2A) mediated acetylcholine release from the epithelium.

In addition to enhancing contraction, TNF-α can influence the synthetic profile of ASM by increasing eotaxin-1 generation along with IL-1β (Ghaffar *et al.*, 1999). The enhancement of calcium transients in human ASM by TNF-α can lead to proliferation, a feature of airway remodelling. The effect was specific to the TNF-α-p55 receptor as TNF-α-p75 specific agonists had no effect (Amrani *et al.*, 1996). It may play a role in severe refractory disease as development of antagonists show it may improve quality of life and numerous end-points but only in a small subgroup of patients (Brightling *et al.*, 2008). Part of the benefit of antagonism in severe refractory disease may be the inhibition of neutrophilia into the lungs, a key symptom of severe chronic asthma (Makwana *et al.*, 2012). Exceeding the therapeutic window for TNF-α inhibition (via a specific therapy or with glucocorticoids) could be serious and lead to a detriment in the body's immunological response, therefore combined use with other treatments could be beneficial (Thomas, 2001).

1.3 Airway smooth muscle

In addition to providing airway tone, the airway smooth muscle (ASM) in healthy patients has numerous other proposed functions including: a developmental role (rhythmic contractions *in utero* assist nutrient supply and waste removal and are necessary for lung development), structural support, protective to noxious stimuli, remodelling to prevent severe bronchoconstriction, production of anti-inflammatory mediators and ventilation perfusion matching (Mitzner, 2004; Pandya *et al.*, 2006; Schittny *et al.*, 2000). However the evidence for these roles after birth are weak at best. Nevertheless, ASM is clearly of great importance in asthma, when its function has been shown to be altered (Benayoun *et al.*, 2003; Hirota *et al.*, 2009; James *et al.*, 2012; James *et al.*, 2008; Wright *et al.*, 2012b). This has led to the use of bronchial thermoplasty to treat asthma by effectively removing or disabling ASM. Three randomised, controlled trials show that bronchial thermoplasty can improve quality of life scores, decrease emergency hospitalisation and decrease mild to severe exacerbations (Thomson *et al.*, 2012). Controversy still surrounds its use as long term safety beyond five years is still unknown and the benefit above long term bronchodilators is debateable (Barnes, 2012). Its benefit however in severe asthmatics provides further evidence of the role ASM plays in the pathophysiology of asthma. The conducting airways provide the

majority of airway resistance in breathing and this is the target area of the treatment, usage in patients with poor pharmacological control or drug adherence may see the greatest benefits (Cox *et al.*, 2004).

Despite the controversy over the role of ASM in the healthy adult population, the evidence is clear concerning its involvement in the pathology of asthma. It is therefore important for the development of new therapeutics to understand fully the role it plays in disease and how the cell phenotype may be altered in asthma.

1.3.1 Role in asthma

ASM cells play a central role in contributing to the asthmatic phenotype. Not only is there more present, as a multicentre analysis of post-mortem tissue observed increased smooth muscle layer thickness in fatal asthmatics compared to controls (James *et al.*, 2008), but they are also functionally different. Asthmatic ASM cells are hypercontractile (Matsumoto *et al.*, 2007), more proliferative (Johnson *et al.*, 2001) and tend to secrete more and/or different mediators (Chan *et al.*, 2006; Hirst, 2003). The excessive contraction is of greatest importance when considering asthma, as Poiseuille's equation states that a halving of lumen diameter will increase airway resistance by a fold of sixteen. Therefore any excessive airway narrowing will significantly increase airway resistance. Other changes in the ASM such as enhanced proliferation and the secretion of inflammatory mediators subsequently results in longer-term changes and remodelling of the lung with further increases in airway resistance (see sections 1.3.3 and 1.4).

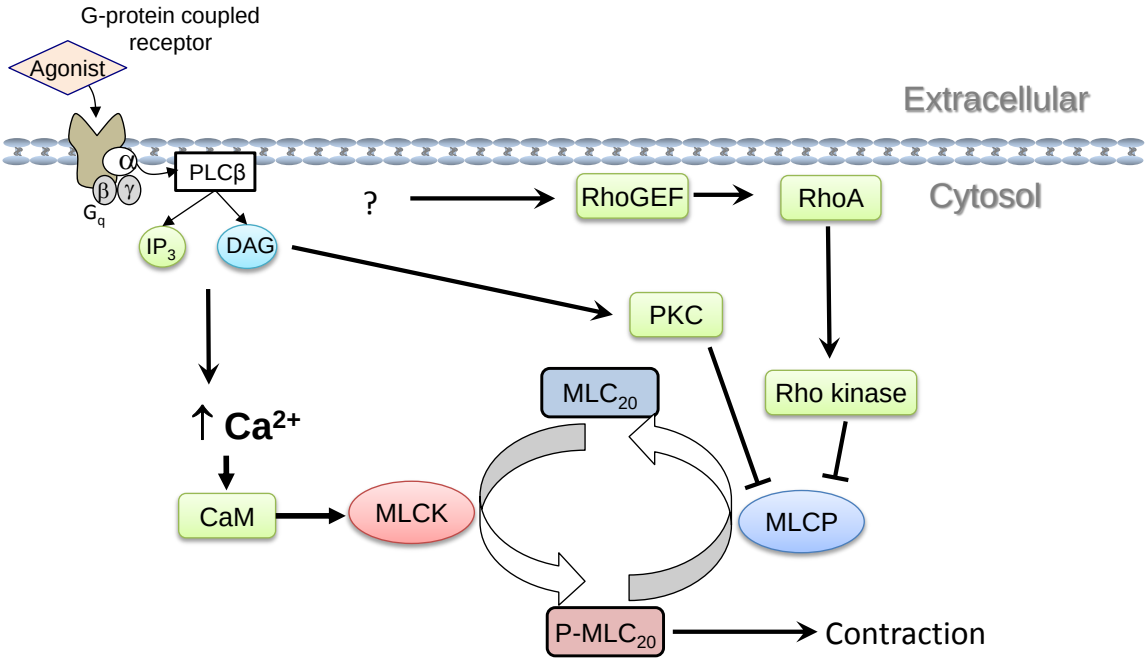
1.3.2 Contractile function

Notwithstanding the discussion of its purpose above, the primary role for ASM is to generate tone and contraction thus regulating the diameter of the airways. The key proteins involved in this process are myosin and actin as well as the regulatory proteins tropomyosin, caldesmon and calponin. Acetylcholine is the endogenous neurotransmitter controlling airway tone, and is released from vagal parasympathetic nerve endings to bind to M_3ACh receptors on the ASM, other bronchoconstrictor agonists of particular relevance to asthma, include histamine, prostaglandin D_2 and cysteinyl leukotrienes. Their receptors are commonly coupled to $G_{\alpha q}$ which activates phospholipase C (PLC), stimulating the formation of diacylglycerol (DAG) and IP_3. The former activates receptor operated calcium entry (ROCE, see section 1.7) and protein kinase C (PKC), whereas the latter initiates release of calcium from intracellular stores (Fig. 1.3.4-1). Many receptors also couple to $G_{12/13}$ with activation of the RhoA/Rho kinase pathway which can induce calcium sensitisation (see below).

Upon a rise in intracellular calcium it forms a complex with calmodulin, which then activates myosin light chain kinase (MLCK). The kinase then phosphorylates the regulatory subunit of myosin (MLC_{20}) allowing the myosin head to bind to actin and initiate it's ATPase activity providing the energy required for contraction. Counteracting MLCK is myosin light chain phosphatase (MLCP) which acts by dephosphorylating myosin to inhibit the cross-bridge cycling and cause relaxation (Chiba *et al.*, 2010). The net activity of both of these enzymes determines the phosphorylation state of MLC_{20} and degree of force production (Sanderson *et al.*, 2008).

Asthmatics exhibit a hypersensitivity to allergens and this is in part due to calcium sensitisation in ASM cells resulting in enhanced bronchoconstriction. Calcium sensitisation is the contraction of smooth muscle via a calcium independent pathway and it is thought that this process is primarily mediated by the RhoA/Rho Kinase (ROCK) pathway (Fukata *et al.*, 2001). Rho kinase phosphorylates the MYPT1 myosin-binding subunit which when phosphorylated inhibits the enzymatic activity of MLCP, resulting in prolonged phosphorylation of the myosin light chain and contraction (Chiba *et al.*, 2010). Both calcium dependent and independent contraction pathways are outlined in Fig. 1.3.2-1.

There also appears to be effects of disease on the calcium sensitisation pathway. It has been shown that TNF-α can induce calcium sensitisation in guinea-pig bronchial smooth muscle independently to MAPK and sphingomyelinase, a potential mediator of TNF-α (Parris *et al.*, 1999**).** Furthermore, the contribution of Rho kinase in ACh induced bronchoconstriction is increased following repeated allergen challenge in rats (Chiba *et al.*, 1999). Inhibition of Rho kinase with the compound Y-27632 has been shown to reverse airway hyperresponsiveness (AHR) to both histamine and prostaglandin $F_{2\alpha}$ following allergen induced early and late-phase responses in ovalbumin sensitised and challenged guinea-pigs (Schaafsma *et al.*, 2006). Together this data shows that the calcium sensitisation pathway may provide novel targets for inhibition of allergen induced bronchoconstriction.



1.3.2-1 An overview of calcium dependent and independent (sensitisation) contraction in ASM both of which are altered in asthma. Calcium sensitisation occurs through inhibition of myosin light chain phosphatase resulting in prolonged phosphorylation of the regulatory light chain MLC_{20}. It is believed that RhoGEF and RhoA both play important roles in this process and may be altered in asthma.

There are many facets to asthma but ultimately it is the narrowing of the airways that causes the majority of the symptoms. Abnormalities of ASM mass and function has been identified as one of the main causes of this (Martin *et al.*, 2000) which is why it is so central to much research and the current mainstay therapy is bronchodilation. The β_2-AR agonists bind to the receptor coupled to $G_{\alpha s}$ which in turn activates adenylyl cyclise resulting in cAMP production and protein kinase A (PKA) activation. PKA can phosphorylate many targets of which the net effect is to reduce intracellular calcium as well as oscillation frequency and inhibit the contractile machinery (decreasing Ca^{2+} sensitivity) (Bai *et al.*, 2006; Knox *et al.*, 1995).

The G-protein G_i may also play a role in mediating contraction and relaxation in ASM cells in asthma. Animal models of asthma have shown increased expression of G_i and was thought to mediate enhanced contraction through G_q and attenuate relaxation through G_s (Hakonarson *et al.*, 1995). However further work in mice has shown that overexpressing G_i decreased contractility of the airways in a proposed protective mechanism while also inferring β_2-AR resistance (McGraw *et al.*, 2007).

Actin polymerisation plays a key role in contraction as its inhibition results in a reduction in force development in tracheal smooth muscle (Mehta *et al.*, 1999) and its expression is increased alongside maximal tension development following repeated allergen challenge (McVicker *et al.*, 2007). Interactions

between the actin cytoskeleton and the ECM through integrins and adhesion junctions enable the transmission of force across the tissue determining (in part) airway compliance and responsiveness (Zhang *et al.*, 2008b). Furthermore, focal adhesion kinase, a non-receptor tyrosine kinase forming part of the adhesion junctions has been shown to phosphorylate Rho GEFs which may lead to calcium sensitisation via the Rho kinase pathway (Somlyo *et al.*, 2003). Cytoskeletal reorganisation within the ASM, also termed "mechanical plasticity" is probably an essential component of ASM function in order to maintain airway lumen diameter (Halayko *et al.*, 2003). It has been postulated that alterations in the process, possible by Rho kinase or interactions with the ECM via integrins as previously mentioned, might underlie the absence of a bronchoprotective effect of deep inspiration as observed in asthma (King *et al.*, 1999).

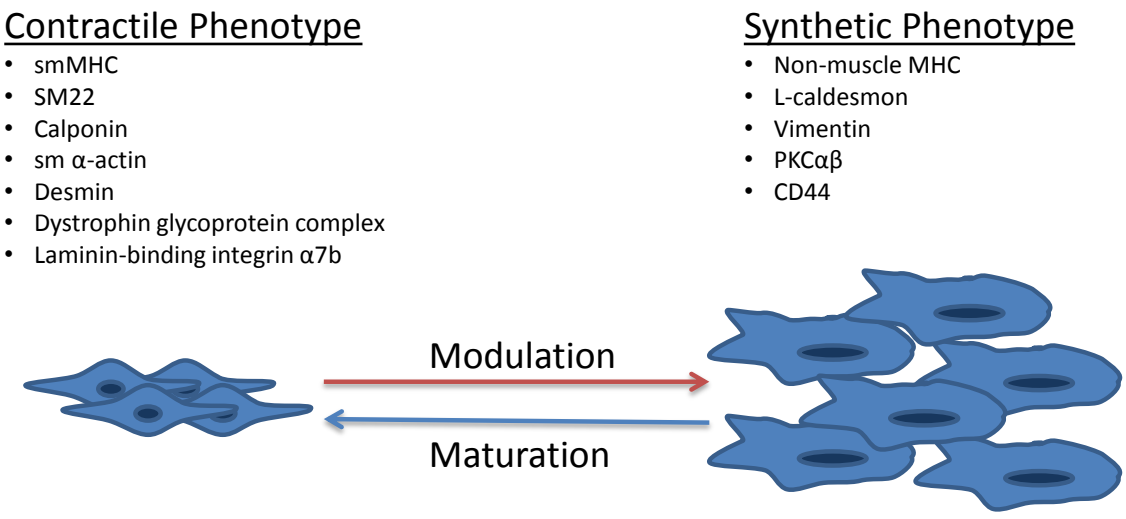
1.3.3 Synthetic function

Alongside the classical contractile function of ASM they have been shown to contribute to the inflammation present in the lungs by secreting cytokines, chemokines and growth factors which recruit inflammatory cells into the airways and the surrounding parenchyma as well as inducing glucocorticoid insensitivity (Tliba *et al.*, 2009). The synthetic function of ASM can be triggered or enhanced by the presence of an allergen, microbe or other antigen. A combination of IL-4, IL-13 and TGF-β, all of which are up regulated in asthma, differentially induces the expression of eotaxin-1 over eotaxin-3 from ASM (Zuyderduyn *et al.*, 2004). Eotaxin-1 serves as a chemokine for eosinophils showing how the ASM can contribute towards bronchoprotection to foreign stimuli. RANTES (regulated upon activation, normal T-cells expressed and secreted) release can be stimulated in culture by TNF-α and IL-1β and can chemo-attract eosinophils, memory T-cells and monocytes (Hershenson *et al.*, 2008). As mentioned previously IL-8 can be secreted by ASM as well as IL-6, monocyte chemotactic protein 1-3, GM-CSF, IL-1β, IL-11 and IFNβ (Panettieri, 2003). In response to airway inflammation adhesion molecule expression is induced on ASM cells, namely ICAM-1 and VCAM-1 allowing leukocytes to adhere to the muscle and possibly aid in activating them on top of inducing ASM proliferation (Lazaar *et al.*, 1994; Panettieri, 2003). The overall effect of these synthetic processes are an alteration of the muscle phenotype to that seen commonly in asthma such as enhanced contractility and perturbed calcium homeostasis (see section 1.5 below) (Amrani *et al.*, 1998; Pepe *et al.*, 2005). These processes have been well defined *in vitro* however the extent of the contribution to the inflammatory response in asthma by the ASM *in vivo* is still to be fully defined.

1.3.4 ASM in culture

As shown, ASM cells display a variety of phenotypes primarily a contractile state (differentiated/maturated) and a synthetic state (dedifferentiated/modulated) (Fig. 1.3.4-1). Evidence from *in vitro* studies are starting to outline that ASM cells can exist in varying degrees between these different phenotypes within a single population and that the characteristics of each phenotype is altered if they are derived from asthmatics (Wright *et al.*, 2012b). It is still unknown whether this phenotypic modulation occurs *in vivo*, but it would go some way to explaining the plethora of responses and functions ASM cells are capable of.

As one might expect, there is a discrepancy between the expressions of certain proteins between both states which contribute to the phenotypic modulation. A rise in synthetic organelles such as the Golgi apparatus and mitochondria aid the increased proliferative capacity of modulated cells (Halayko *et al.*, 2008) while a concomitant fall in contractile markers decrease the cell's responsiveness to contractile agonists (Gosens *et al.*, 2002). This can be induced by addition of different stimuli such as platelet derived growth factor (PDGF), extracellular matrix proteins such as fibronectin and collagen-I (Dekkers *et al.*, 2007).



1.3.4-1 Examples of proteins which differ in expression levels between the modulated and maturated state in cultured ASM cells, redrawn from Wright *et al*., 2012b.

Many factors can initiate a phenotypic change in smooth muscle from serum content of the culture media and confluency of the cells, to supplementation of cytokines and ECM proteins (Halayko *et al.*, 1999), (Hirst *et al.*, 1996). Performing experiments on cells at an early passage is also important, Panettieri et al.

(1989) reported that smooth muscle specific α and γ-isoactin decreases significantly in the third passage. It is therefore very important to design any experiment with ASM carefully and use the appropriate conditions to best test the hypothesis.

1.4 Airway remodelling

Airway remodelling encompasses the structural changes in the lung observed with asthma and is characterised by increased ASM mass, sub-epithelial fibrosis, goblet cell hyperplasia, sub-mucosal gland hypertrophy, neoangiogenesis, epithelial apoptosis, altered ECM and increased activated fibroblasts (Berair *et al.*, 2013). It is generally believed that these structural changes are largely irreversible (Barnes, 1996) and therefore are indicative of disease progression and symptom worsening.

Important work is being carried out to determine the relationship between inflammation, airway remodelling and AHR. It is critical to elucidate which aspect increases airway lumen narrowing the most and the time course in which they are brought on to successfully prevent it. In asthmatic patients who display a late-phase reaction to allergen challenge it has been shown that airway wall cellular inflammation increases at 24 hours and resolves by 7 days post challenge. AHR and markers of remodelling persist up to this point (Kariyawasam *et al.*, 2007), indicating that although inflammation may be required to initiate remodelling and AHR, its presence is not required to sustain it. In an ovalbumin mouse model of airway remodelling it was shown that ECM deposition, particularly collagen, was increased during persistent challenges and did not resolve up to 1 month post cessation (McMillan *et al.*, 2004). An elevated number of ASM cells were also maintained after cessation of challenges while inflammatory markers TGF-β1, IL-4, IL-5 and IL-13 fell back down. Both of these studies further point to the potentially irreversible nature of the remodelling as exposure to antigens or presence of inflammation are not required for their persistence.

It has been suggested that airway remodelling can contribute to AHR as ASM length increases as part of the process (Lee-Gosselin *et al.*, 2013). When human bronchial rings were stretched they produced greater force in response to methacholine and were more sensitive to contractile agonists. Using computational analysis they reasoned that ASM from asthmatics may be increased by up to 10.4% in length. The study relies on numerous assumptions but it provides an interesting link between changes in ASM morphology, (i.e. increased ASM length as a part of remodelling) and AHR. This paradigm can also be turned on its head as it has also been shown that bronchoconstriction alone can induce remodelling (Grainge *et al.*, 2011). The paper demonstrates increased epithelial proliferation, mucus production and collagen thickness

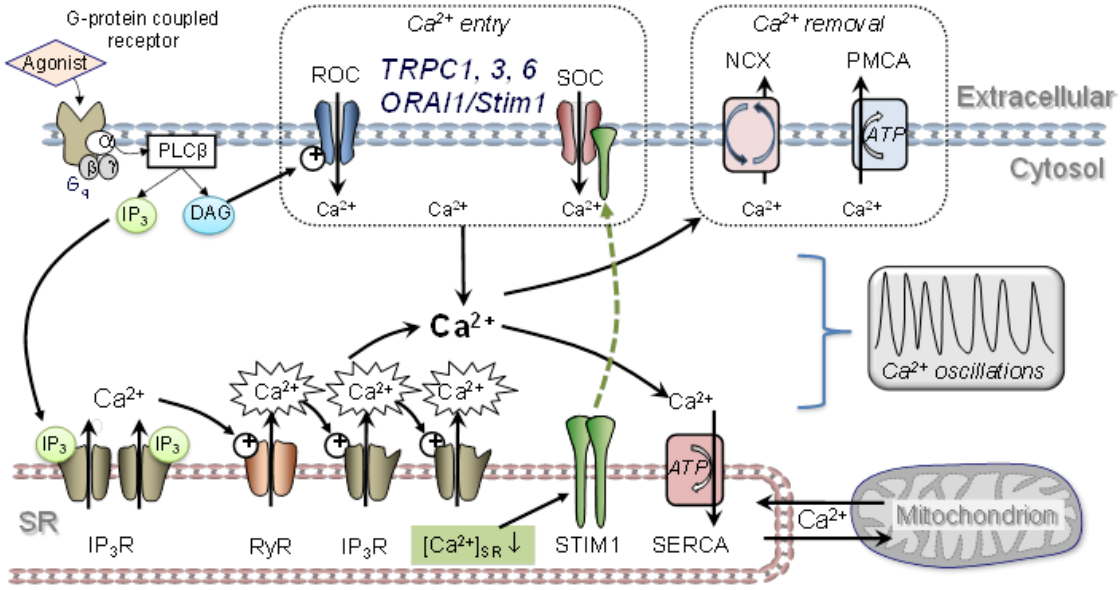
induced by methacholine challenge in asthmatic patients that was prevented by pre-treatment of salbutamol, a β_2-AR agonist. The conclusion draws a parallel to skeletal muscle where repetitive contraction leads to remodelling. Part of their suggestion lies with an increased epithelial stress leading to enhanced TGF-β production in the epithelial-mesenchymal trophic unit which can result in remodelling (Halwani *et al.*, 2011). An interesting follow up study would be to control for the other pharmacological effects of methacholine and salbutamol by stimulating with other contractile agents. Thus ensuring that it is contraction inducing the remodelling and not other receptor mediated effects. Further weight has been added to this theory using guinea-pig lung slices as bronchoconstriction using methacholine induced TGF-β release resulting in airway remodelling in the absence of inflammation (Oenema *et al.*, 2013a).

The increased ASM layer observed in remodelling is thought to be a result of both hyperplasia and hypertrophy however it is largely believed the former is of greater significance. The source of the additional ASM cells is still controversial as there is conflicting evidence over it being from increased proliferation (Johnson *et al.*, 2001; Ward *et al.*, 2008) or prolonged survival (Berair *et al.*, 2013). One current idea is that fibrocytes in the peripheral blood are recruited to the ASM bundle by the ASM itself in asthma (Saunders *et al.*, 2009) and once there, they are able to differentiate into myofibroblasts and eventually ASM (Davies *et al.*, 2003). Another potential source is from the epithelium in a process termed "epithelial mesenchymal transition" where the expression of numerous proteins change resulting in a loss of epithelial functionality and acquisition of a mesenchymal-like phenotype. There is some evidence this may contribute to the airway remodelling observed in asthma using a chronic murine allergen exposure model (Johnson *et al.*, 2011). The paper shows that airway epithelial cells lost expression of E-cadherin and occludin while gaining expression of mesenchymal markers vimentin, α-sm-actin and pro-collagen-I with a concomitant migration into sub-epithelial regions of the airway wall. There is evidence that this process may occur in humans using a similar challenge in primary *in vitro* culture of bronchial epithelial cells (Heijink *et al.*, 2010). There is still some controversy to what degree this process occurs *in vivo* in asthma as a further study failed to show the expression of any mesenchymal markers in epithelial cells from sections taken from asthmatic donors therefore the phenomenon may just be part of the normal epithelial repair process (Hackett *et al.*, 2009).

1.5 Calcium homeostasis

Cytosolic calcium is an important regulator for many intracellular functions including those central to the asthmatic phenotype. Resting cytosolic calcium concentration $[Ca^{2+}]_i$ is approximately 100nM; this is 20,000 times lower than the extracellular concentration and considerably lower than that in the

sarcoplasmic reticulum (SR; 500μM). The low resting $[Ca^{2+}]_i$ is critical to the normal functioning of the cell as numerous mechanisms including contraction, transcription, secretion, migration and proliferation are dependent on subtle changes in its levels (Mahn *et al.*, 2010). Calcium homeostasis is controlled by several proteins on the plasma membrane controlling its entry and removal from the cell and also by proteins on the sarcoplasmic membrane capable of sequestering and releasing intracellular stores (Fig. 1.3.4-1).



1.3.4-1 An outline of intracellular calcium homeostasis in ASM redrawn with permission from Mahn *et al.*, 2010. Upon agonist association with a $G_{\alpha q}$ coupled receptor PLCβ activation generates IP_3 and DAG resulting in a rise in $[Ca^{2+}]_i$ through release from the SR reticulum and receptor operated calcium entry respectively. Calcium release from the SR initiates calcium oscillations by increasing the opening probability of the ryanodine receptor in a process termed calcium induced calcium release (CICR). In each cycle of the oscillations calcium is removed from the cytosol via sequestering back into the SR by SERCA2 or extrusion from the cell either by the sodium calcium exchanger (NCX) or the plasma membrane ATPase pump (PMCA). To replenish the SR calcium content store operated calcium entry is initiated using the "sensing" protein STIM1 in association with Orai1 and the TRPC channels.

Upon $G_{\alpha q}$ receptor activation by an agonist calcium is released from intracellular stores via IP_3 which then increases the opening probability of the ryanodine receptor resulting in further calcium release and the initiation of calcium oscillations (see section 1.5.1 below). The calcium signal causes myosin light chain kinase activation resulting in muscle contraction (see Fig. 1.3.2-1). The subsequent elevation of $[Ca^{2+}]_i$ initiates numerous other responses including activation of pro-inflammatory transcription factors such as NFAT (nuclear factor of activated T cells) and NF-κB, leading to changes in gene expression. The termination of these processes in part relies on the ability of the cell to bring $[Ca^{2+}]_i$ back to resting levels. It can do this either by extrusion of calcium from the cell via the sodium/calcium exchanger (NCX) or the

plasma membrane calcium ATPase pump (PMCA), or by pumping it into intracellular stores via sarcoendoplasmic Ca^{2+} ATPases (SERCA). SERCA provides the sole mechanism for sequestering free cytosolic calcium into the sarcoplasmic reticulum and therefore strongly dictates the rate of recovery to resting levels. A third mechanism involves mitochondrial buffering and is described in further detail in section 1.5.2.

Recent studies have shown that the tight regulation of protein expression controlling calcium homeostasis is controlled to a degree at the transcriptional level, at least in cardiac muscle (Ritchie *et al.*, 2011). Early Growth Reponse-1 (EGR1) can modulate the expression of SERCA2 (Arai *et al.*, 2000), NCX (Wang *et al.*, 2005) and Stim1 (Ritchie *et al.*, 2010) in cardiac myocytes and cell lines. EGR1 is a receptor dependent transcription factor showing the capability for ligands to completely remodel the calcium handling dynamics and potentially alter the cell phenotype.

1.5.1 Calcium oscillations

There is a highly non-linear relationship between $[Ca^{2+}]_i$ and force production in smooth muscle due to the 1:4 stoichiometry of calmodulin-Ca^{2+}. In light of this and because of the large diversity of stimuli which can result in bronchial hyper-reactivity it was postulated a while ago that dysregulation of calcium homeostasis may be a unifying underlying cause (Triggle, 1983). Calcium independent mechanisms contributing to AHR must also be considered, see 1.3.2 above.

In culture near maximum concentrations of contractile agonists cause a biphasic increase of $[Ca^{2+}]_i$ in a population of ASM cells. The first phase is transient and results from intracellular calcium release whereas the secondary phase is sustained and dependent on extracellular Ca^{2+} (Murray *et al.*, 1991). More powerful techniques has since shown the detail of signalling within each individual cell is far more complex. Upon agonist stimulation calcium oscillations initiate from IP_3 associating with its receptor causing SR Ca^{2+} release resulting in an increase in the RyR opening probability as discussed above. The IP_3R also increases its opening probability upon calcium binding to a high affinity site initiating a propagating calcium wave along the cell which is regenerative in nature due to further calcium-induced calcium release from the SR (Liu *et al.*, 1996; Prakash *et al.*, 1997). The oscillations occur due to a second, lower affinity Ca^{2+} binding site on the IP_3R which reduces its opening probability when the $[Ca^{2+}]_i$ is high enough (Iino, 1990). Some Ca^{2+} is lost from the cell through the PMCA pump and the NCX, the signal maintains its regenerative aspect through replenishing the SR via store operated calcium entry (SOCE), see section 1.7.

The frequency and duration of calcium oscillations correlates with agonist concentration and ASM contraction however the amplitude of each oscillation remains relatively constant (Sanderson *et al.*, 2008). The oscillations between cells are asynchronous therefore they do not generate synchronised phasic contractions as observed in some other smooth muscle tissue (Ressmeyer *et al.*, 2010)**.** The current hypothesis is that it is the sum effect of the frequency of all these asynchronous oscillations within each cell that cause tonic contraction in the ASM and that the onset of contraction appears to be almost simultaneous with the onset of calcium oscillations upon agonist stimulation.

It is thought that the mean global cellular rise in calcium ions, integrated from many local oscillations, plays a role in signalling for slower Ca^{2+} dependent processes (Prakash *et al.*, 2000) as well as their frequency. The oscillations usually start from one side of the cell and propagate along its entire length. In calcium free conditions agonists can initiate oscillations but not sustain them longer than a few cycles showing that calcium influx is required to fill the intracellular stores (Prakash *et al.*, 1997; Sanderson *et al.*, 2008). The time taken to do this, which is in part dependent upon SERCA2 activity, can dictate oscillation frequency (Sneyd *et al.*, 2004) and therefore potentially affect the functional outcome. The importance of store release in this process is highlighted by the fact SERCA2 inhibition results in a rundown of store content and reduces oscillation frequency (Perez-Zoghbi *et al.*, 2007). This paper also showed the positive correlation between oscillation frequency and contraction, indicating this is in part how contraction is regulated by different agonist concentrations. It is thought that agonist induced oscillations involve both IP_3R to initiate the response and determine the baseline characteristics and cyclic ADP-ribose (cADPr) acting via RyR to modify the response, although it is not a requirement for it (Sanderson *et al.*, 2008). cADPr is a nucleotide metabolite whose synthesis and degradation is catalysed by CD38 in ASM and has also been suggested to alter calcium homeostasis during inflammation, reviewed in (Deshpande *et al.*, 2005).

High resolution microscopy has revealed that calcium oscillations have distinct spatial characteristics which add another level of informational coding. It has been postulated that a superficial buffer barrier exists in which components of the sarcoplasmic reticulum divide the cytosol into at least two discrete signalling spaces. Thus serving to create a variable calcium gradient immediately below the plasmalemmal membrane and also acting as an amplifier of signalling via calcium induced calcium release (CICR) (Janssen *et al.*, 1999; van Breemen *et al.*, 1995).

1.5.2 Mitochondria

Mitochondria are essential to life, producing adenosine triphosphate (ATP) which is required to provide energy for numerous cell functions. It is also involved in protein, steroid and lipid synthesis as well as cellular signalling cascades (McBride *et al.*, 2006). Mitochondria has also been shown to play a role in Ca^{2+} handling, helping to shape calcium signalling patterns and buffer high concentrations to prevent excitotoxicity (Carafoli, 2010; Parekh, 2003; Vasington *et al.*, 1962).

Mitochondria in conjunction with the SR divides the cytosol into distinct microdomains as either can occur both peripherally or centrally within the cell allowing an extra depth to calcium signalling (Rizzuto *et al.*, 2006). Its close proximity with both the SR and plasma membrane allows the mitochondria to shape the $[Ca^{2+}]_i$ response to agonists via its buffering function (Delmotte *et al.*, 2012). By actively up taking calcium from the cytosol it has been shown that mitochondria increase the dynamic range of IP_3 induced SOCE (see section 1.7) and could therefore play a role by lowering its activation threshold (Gilabert *et al.*, 2001). Further manipulation of SOCE is possible by the mitochondria as suggested by a study using calcium indicators specific for the ER lumen, mitochondria and cytosol (Arnaudeau *et al.*, 2001). By systematically preventing uptake into either the SR or mitochondria with antagonists and stimulating the cell with histamine the paper postulates that the mitochondria can locally refill areas of the SR creating internal local calcium gradients. A benefit of this could be allowing the plasmalemmal SR to deplete and activate SOCE, while replenishing the internal SR to continue cytosolic calcium oscillations for contraction and nuclear signalling.

Interestingly, treatment with the inflammatory cytokine TNF-α causes an increased rise in $[Ca^{2+}]_i$ in response to agonist stimulation, while causing a decrease in $[Ca^{2+}]_{myt}$ by inhibiting the mitochondrial uniporter (MCU) (Delmotte *et al.*, 2012) which uptakes calcium from the cytosol (De Stefani *et al.*, 2011). Calcium levels within the mitochondria also act to modify catabolic processes by stimulating the dehydrogenases; pyruvate (Denton *et al.*, 1972), iso-citrate (Denton *et al.*, 1978) and 2-oxoglutarate (McCormack *et al.*, 1979). Thus mitochondria can affect cellular calcium signalling and subsequent changes in calcium within the mitochondria can affect metabolism. Changes in the mitochondria have been shown to be relevant in asthma as ASM have an increased mitochondrial mass and oxygen consumption compared to both COPD patients and healthy controls (Trian *et al.*, 2007). The increase in mitochondrial mass is a result of a calcium dependent increase in mitochondrial biogenesis which in turn lead to a mitochondrial dependent increase in asthmatic ASM compared to the other groups. The findings

presented show that both the mitochondria or the altered calcium handling in ASM (discussed in detail in section 1.6) could be used as therapeutic targets for airway remodelling in asthma.

1.5.3 Lysosomal calcium stores

The complexity of calcium signalling is further enhanced by the interaction of the endoplasmic reticulum with lysosomal calcium stores. They are IP_3 and RyR independent and release Ca^{2+} in response to NAADP, originally found in sea urchin eggs (Churchill *et al.*, 2002). It has been postulated that NAADP and cADPR may link the cellular metabolic state to calcium signalling mechanisms via this store (Berridge *et al.*, 2003), as can the mitochondria, however there is no clear role described currently in ASM.

1.6 Calcium handling proteins

1.6.1 SERCA2

There are three members of the SERCA family each with different splice variants, in the ASM SERCA2b is the most highly expressed (Mahn *et al.*, 2009). There are many proteins involved in regulating the activity and expression of SERCA2b with much of the research to date being focussed on its role in cardiac function. Phospholamban is the most studied regulator of SERCA2 and in its unphosphorylated form it lowers the affinity of SERCA for calcium. In the heart clear links have been shown between super or chronically active phospholamban inducing dilated cardiomyopathy (MacLennan *et al.*, 2003). Pro-inflammatory cytokines in the asthmatic lung lead to increased agonist induced $[Ca^{2+}]_i$ so a natural presumption would be dysregulation of intracellular calcium homeostasis at some level and it was hypothesised that phospholamban expression or activation may be increased. However it was shown that human ASM does not express the phospholamban protein (Sathish *et al.*, 2009) and that its regulation may involve CaMKII. The same paper also reports pro-inflammatory cytokines IL-13 and TNF-α reducing SERCA expression upon incubation overnight. Further data shows TGF-β reducing SERCA expression for up to 7 days post 24 hour incubation with ASM cells (Ojo,O., Thesis, 2011, University of London). Both of these sets of data can help to describe why chronic inflammation which is present in asthma can lead to disruption of calcium homeostasis.

Impaired function of SERCA2 matches its reduced expression *in vitro* as stimulation of asthmatic ASM cells by bradykinin or ryanodine resulted in an attenuated rise in $[Ca^{2+}]_i$ and a subsequent slower recovery to baseline compared to healthy controls (Mahn *et al.*, 2009). Further evidence is given to the importance of SERCA in asthma by reducing its levels using targeted siRNA. The paper shows that by reducing

SERCA2 in the healthy cells it is possible to reproduce many of the phenotypes shown in asthmatic cultured cells such as enhanced secretion of inflammatory cytokines, proliferation and motility. Mahn *et al* have therefore provided compelling evidence that reduced expression of SERCA2 is present in asthmatic airways and that the level of reduction can have functional consequences in ASM cells in culture.

The SERCA pump is an ATPase and requires ATP to function which is produced by the mitochondria. Efficient energy production and expenditure may be regulated at a transcriptional level and it has been shown the mitochondrial transcription factors A (TFAM) and B2 (TFB2M), which are key regulators of ATP production also regulate nuclear transcription of SERCA2 (Watanabe *et al.*, 2011). The latter report also demonstrated that the transcription factor SP1, which is known to regulate SERCA2 transcription, has a very close binding site to TFAM though they act independently. TFAM and TFB2M mRNA levels are decreased in the failing heart (a disease in which SERCA2 is also decreased) and their expressions are in part controlled by the co-factor peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) (Watanabe *et al.*, 2011). PGC-1α has been shown to be vital for the heart to meet increasing demands for ATP and in mice lacking it cardiac dysfunction occurs (Arany *et al.*, 2005). It has been postulated that stimulation of PGC-1α via PPAR-γ agonists could provide a new therapeutic approach (Sano *et al.*, 2005). Given the close pathology of calcium homeostasis in heart failure and asthma this could be an avenue worth investigating.

1.6.2 ORMDL3

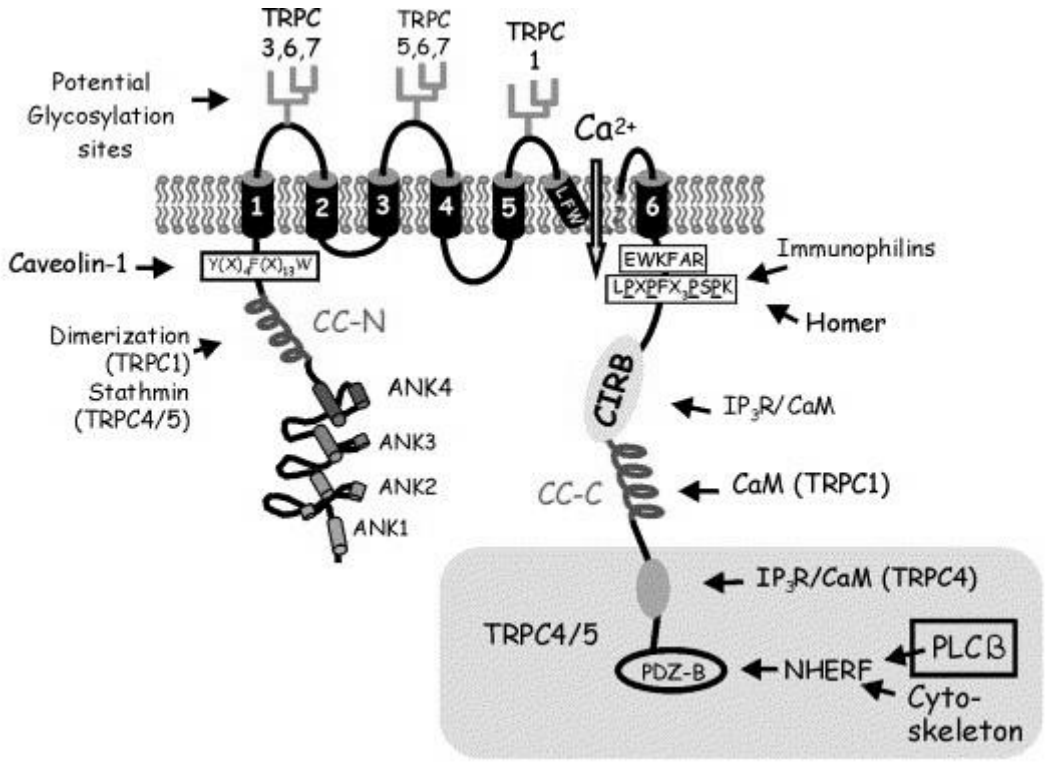
There is a strong association with the onset of childhood asthma and a single nucleotide polymorphism (SNP) on chromosome 17q21 relating to ORMDL3 transcript levels (Moffatt *et al.*, 2007). The polymorphism has been specifically associated in asthma without rhinitis and found to be also linked to COPD patients (Balantic *et al.*, 2013). ORMDL3 is expressed on the ER which has been shown to have an important role in sensing cellular stress via the unfolded protein response (UPR) (Zhang *et al.*, 2008a). ORMDL3 can alter calcium homeostasis in the ER and facilitate the UPR possibly by binding to and inhibiting SERCA2b (Cantero-Recasens *et al.*, 2010). It is unknown why the ORMDL3 SNP is only associated with childhood asthma however this could possibly be due to hormonal effects counteracting its perturbation of calcium homeostasis.

1.6.3 TRPC

The mammalian transient receptor potential (TRP) channel proteins consist of six families containing TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin) and

TRPML (mucolipin). The TRPC subfamily is further divided into seven members, TRPC1-7. Each member of the subfamily contains six transmembrane spanning domains and assemble as homo or heterotetramers to form cation selective channels (Pedersen *et al.*, 2005). TRPC3, TRPC6 and TRPC7 are grouped together as they share a higher degree of sequence homology compared to the other four as well as forming multiple heteromers with each other (Cheng *et al.*, 2010; Hofmann *et al.*, 2002) and being responsive to DAG, making them receptor operated calcium channels (ROC). TRPC1, C3, C4 and C6 are expressed in human ASM (Corteling *et al.*, 2004; Karner, 2010). There is also evidence TRPC7 is expressed at low levels in a portion of the population (Karner, 2010). Both TRPC3 and TRPC6 can be activated by DAG in a membrane delimited fashion (Hofmann *et al.*, 1999).

It has been shown that TRPC3 is less tightly regulated and has higher constitutive activity than TRPC6 due to the N-linked glycosylation pattern. Evidence shows that TRPC6 is dually glycosylated within the first and second extracellular loop whereas TRPC3 is monoglycosylated (Dietrich *et al.*, 2003), see Fig. 1.6.3-1. In a further publication by the group they back up this suggestion by showing increased expression of TRPC3 leads to increased basal calcium current and knocking TRPC3 with siRNA in $TRPC6^{-/-}$ cells returned the enhanced basal and response to OAG back to wild type levels. The paper proposes they form dimmers in which TRPC6 regulates the basally active TRPC3 (Dietrich *et al.*, 2005). Increasing expression of TRPC3 in wild type cells or reducing TRPC3 in the TRPC6 knockouts with siRNA abolished the increased cation entry, leading to the conclusion that both channels are functionally non-redundant and TRPC6 is required to regulate muscle tone.



1.6.3-1 Structural features of TRPC channels (Vazquez *et al.*, 2004a). Potential glycosylation sites are labelled. The 70kDa TRPC6 splice variant terminates at the N-terminus mid-way through the coil-coiled domain. The two larger truncated splices of the full length TRPC6 are missing the first hydrophobic segment of the protein.

TRPC6 channels are non-selective cation channels allowing the entry of calcium and sodium ions into the cell (Godin *et al.*, 2007). It is thought that the sodium entry through TRPC6 results in membrane depolarisation and activation of voltage dependent L-type calcium channels or the sodium calcium exchanger (NCX) (Cloutier *et al.*, 2003; Eder *et al.*, 2005). The Godin 2007 paper shows that reducing TRPC6 with siRNA did not alter OAG induced intracellular Ca^{2+} movement in primary cultured ASM from guinea-pigs. They suggest that TRPC3 homotetramers mediate the OAG response in the absence of TRPC6 after siRNA treatment. Unfortunately the paper does not inhibit or knockdown NCX or L-type calcium channels as one would expect TRPC6 would still be contributing to calcium entry following OAG stimulation indirectly via one of these two entry mechanisms following Na^{+} influx. Such an additional experiment would help delineate the contribution of calcium or sodium ion entry via TRPC6 on the net calcium influx. Their data also contradicts previous studies in our lab showing TRPC6 siRNA administration significantly ablates the OAG response as analysed by 100s analysis of variance (ANOVA) (Karner, 2010).

There are two TRPC3 splices, a shorter form missing some of the N-terminus lacking from an upstream exon weighing 97.37 kDa and a longer isoform at 105.55 kDa. The number of functional TRPC6 splices is contentious, there are likely to be three or four**.** There was no evidence of TRPC3 or TRPC6 splice variants expressed in murine or canine pulmonary or renal artery smooth muscle preparations reported in a previous publication (Walker *et al.*, 2001). Very little work has been done so far on profiling the expression and function of the various TRPC splice variants' expression in ASM. It would be of great use to quantify this expression and gain a clearer idea of which splices assemble into dimers with each other and how that translates into channel coupling and function.

The roles of TRPC3 and TRPC6 in calcium entry are contentious and discussed in further detail in section 1.7. An obligatory role of Src kinases has been found in DAG activation of TRPC3 in HEK293 cells (Vazquez *et al.*, 2004b). A dominant negative form of Src kinase completely suppressed receptor and OAG dependent TRPC3 activation. SOCE however was unaffected and shown not to be Src dependent, in contrast to other findings in fibroblasts and platelets (Babnigg *et al.*, 1997; Rosado *et al.*, 2004). Findings made in cell lines must be interpreted with caution, particularly when considering function of TRPC channels as they are influenced by so many different factors.

Neither TRPC3 nor TRPC6 message RNA expression is altered in ASM cells derived from a range of severities of asthma compared to control (Karner, 2010). The asthmatic cytokine TNF-α has been shown to increase TRPC3 expression and SOCE thought to be associated with it (White *et al.*, 2006). The lack of changes observed in mRNA may be a result of the inflammatory cytokine stimulus being removed and therefore is not necessarily representative of the *in vivo* situation. The combination of an increased expression of TRPC3 and decrease in SERCA2 following stimulation with pro-asthmatic cytokines means a relatively severe disruption of calcium homeostasis is likely to exist in asthmatic ASM cells. What is yet to be shown though is whether this has any significant consequences in a whole organism and if this can be manipulated into an animal model of asthma.

The lack of specific antibodies, agonists and antagonists makes the study of TRPC proteins particularly difficult. Additionally their complex dimerisation and association with other proteins such as Orai1 add to their functional diversity making it difficult to elucidate the role of each TRPC protein. Their role in Ca^{2+} influx in ASM and the link to contraction and proliferation make them a potentially valuable target for asthma and warrants perseverance in their research (Perez-Zoghbi *et al.*, 2009).

1.6.4 Na^+/Ca^{2+} exchanger

Upon agonist stimulation and a rise in $[Ca^{2+}]_i$ removal mechanisms are activated to bring the concentration back to basal levels as previously discussed by SERCA2 (see 1.6.1). An additional mechanism is via the Na^+/Ca^{2+} exchanger (NCX) which utilises the trans-membrane Na^+ gradient to efflux one calcium ion in exchange for three sodium ions which is an electrogenic process. The transporter can also work in reverse to pump calcium ions into the cell, a phenomenon described to occur in ASM mediated by STIM1 to help control calcium homeostasis (Liu *et al.*, 2010). The close association of NCX with calsequestrin containing regions of the SR (Moore *et al.*, 1993) and potentially TRPC3 (as shown in HEK293 cells) (Rosker *et al.*, 2004) further suggests its role in calcium homeostasis (Sathish *et al.*, 2011).

The most comprehensive study of NCX in human ASM within the context of asthma showed that only NCX1 and not NCX2-3 are expressed at the protein level (Sathish *et al.*, 2011). The cytokines TNF-α and IL-13 both significantly elevated its protein expression in an NF-κB and MAPK dependent manner. Importantly, treatment with the cytokines increased the inward (often termed reverse mode) exchange mode of the pump to a greater degree than the outward exchange mode and inhibition of its expression decreased both the peak and rate of fall of $[Ca^{2+}]_i$ following histamine stimulation. The paper provides clear evidence how the NCX pump may play a role in the altered calcium homeostasis in asthma.

1.7 Store and receptor operated calcium entry

1.7.1 SOCE

Store operated calcium entry (SOCE) also known as capacitative calcium entry (CCE) involves the refilling of the sarcoplasmic reticulum store of calcium from the extracellular fluid following agonist induced emptying (Putney, 1986). As stated previously, this entry is required to sustain calcium oscillations which are critical in cellular signalling (Prakash *et al.*, 1997; Sanderson *et al.*, 2008).

Numerous mechanisms have been postulated since the discovery of this phenomenon including the vesicular fusion of store operated channels with the plasma membrane via exocytotic machinery, a diffusible messenger (such as Ca^{2+} influx factor) or the conformational coupling of the IP_3 receptor to the plasma membrane (Parekh *et al.*, 2005). Mounting evidence has since shown Stromal interaction molecule 1 (STIM1), the Orai group of proteins and the TRPC channel family mediate this effect (Feske *et al.*, 2006; Liao *et al.*, 2007; Peel *et al.*, 2008; Putney, 2005; Zhang *et al.*, 2005).

Stromal interaction molecule 1 (STIM1) acts as a Ca^{2+} sensor on the SR and translocates to the plasma membrane upon store depletion (Zhang *et al.*, 2005) where it interacts with Ca^{2+} permeable channels. It is

comprised of a single membrane spanning domain and a helix-loop-helix motif (EF hand) close to the luminal C-terminus which acts as the calcium sensor. It is thought that STIM1 interacts with Orai1, a plasma membrane protein containing four transmembrane segments (Feske *et al.*, 2006). The paper shows that a homozygous single missense mutation in severe combined immune deficiency patients leads to the lack of SOCE. In turn there is no activation of nuclear factor of activated T-cells (NFAT) transcription giving rise to the phenotype. In a smooth muscle specific knockout of STIM1 this finding was backed up as PDGF failed to activate NFAT in the knockout mice as was sustained PDGF induced Ca^{2+} entry (Mancarella *et al.*, 2013).

It was originally thought that Orai1 forms a pore through the channel but recent evidence has shed doubt on the theory and proposed that it combines with TRPC3 or TRPC6 to form a regulatory subunit (Liao *et al.*, 2007). The interactions between Orai1 and TRPC channels would dictate their ability to perform both ROCE and SOCE depending upon their expression and perhaps presence in lipid rafts (Liao *et al.*, 2009).

Caveolar expression plays a role in SOCE in ASM cells, when caveolin is knocked down with siRNA Ora1 expression is also reduced with a concomitant decline in SOCE (Sathish *et al.*, 2012). The paper also demonstrates how SOCE can be altered by inflammation in asthma as exogenous application of TNF-α increased SOCE. Part of the mechanism behind this could be increased STIM1 aggregation resulting in greater puncta size (Jia *et al.*, 2013).

1.7.2 ROCE

ROCE is defined as the portion of calcium entry into the cell which is attributed to the continued presence of agonist and can be abolished experimentally by an antagonist of the receptor. The influx of Ca^{2+} is independent to the SR Ca^{2+} content, unlike SOCE and are mediated by TRPC channels. It has been shown that ROCE occurs in untreated ASM cells upon stimulation with ACh and that treatment with TNF-α causes a switch to a more SOCE dominated calcium entry mechanism (White *et al.*, 2006). The switch in function is coupled with an increase in TRPC3 expression which decreases ion channel coupling to PLC and increases its sensitivity to store depletion. Very little work has been done to date to separate the functions of SR sensitive and insensitive calcium entry through TRPC channels and they may prove to serve a role in regulating a change in calcium signalling in the presence of inflammation.

1.8 TRPA1

TRPA1 is an excitatory ion channel located on sensory neurons and is activated by numerous agonists including; mustard oil, garlic, reactive oxygen species and cigarette smoke (Bautista *et al.*, 2006; Caceres

et al., 2009; Jordt *et al.*, 2004). TRPA1 activation can lead to neurogenic inflammation in the lung (Andre *et al.*, 2008) and it was found when ablating its protein expression using a homozygous knockout, ovalbumin induced leukocyte infiltration, mucus production, cytokine infiltration and bronchial hyperreactivity were all dramatically reduced (Caceres *et al.*, 2009). The results of this study were all put down to its neurogenic inflammation however it has since been found to be able to contribute to non-neuronal inflammation in mice (Nassini *et al.*, 2012).

Current respiratory research into TRPA1 is centred primarily on its role in the tussive response to both exogenous and endogenous agents (Grace *et al.*, 2012). In light of the recent finding that it is expressed on numerous cells including cultured ASM, fibroblasts and epithelial cells new mechanisms contributing to asthma and other respiratory diseases via altered cation entry may be discovered.

1.9 Current therapies

1.9.1 β_2-adrenergic receptor agonists

β_2-adrenergic receptor (β_2-AR) agonists form a vital part of many asthmatic's mainstay treatment as they offer a quick onset of action relaxing ASM and they have a good safety profile. The agonist shifts the equilibrium of the receptor in favour of its activated form from its resting inactive state thereby increasing the number of receptors associated with the G_s protein and GTP (Johnson, 2001). The activated receptor results in numerous downstream signalling events but importantly an increase in cAMP followed by activation of PKA results in muscle relaxation.

The first generation of these drugs including salbutamol only have a maximum duration of action of four hours. Therefore long acting β_2-AR agonists (LABA) were developed particularly for patients whom attacks were worst at night or morning. LABAs are able to elicit a prolonged response compared to other drugs of the same class, for example salbutamol, because of their enhanced lipophilicity and potential enhanced dissociation constant with the receptor (Lotvall, 2001). The increased lipophilic nature of the compounds results in them staying in the phospholipid bilayer of the cell membrane and reaching the receptor in a prolonged manner and are not washed away so easily as hydrophilic compounds.

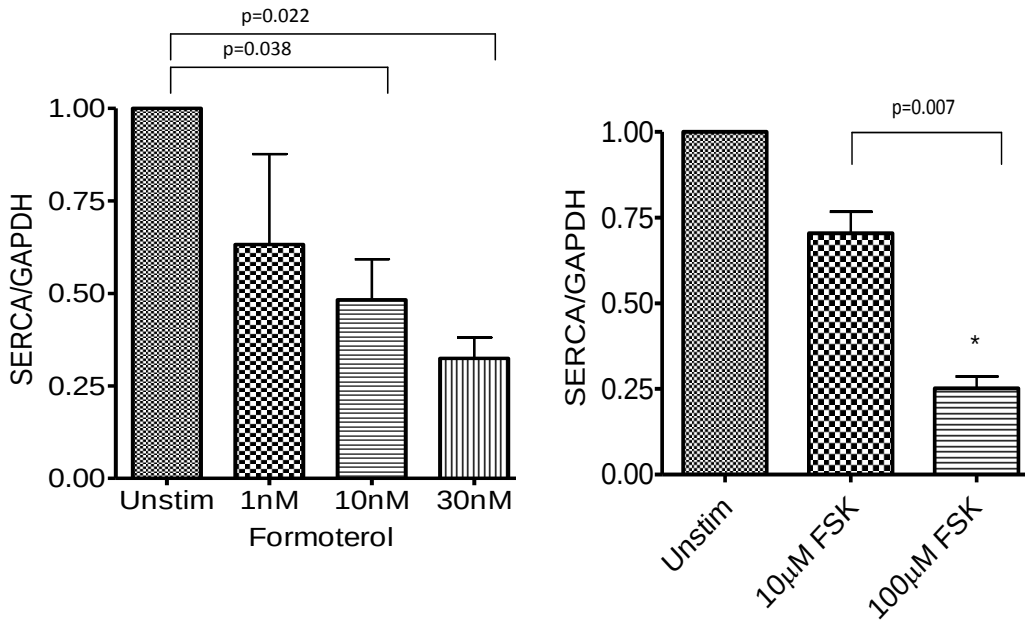
1.9.2 Postulated limitations of LABAs

The safety of formoterol and other LABAs has been called into question following increasing evidence showing an increased risk of mortality (Rodrigo *et al.*, 2009). The β_2-AR has constitutive activity in the absence of an occupying agonist (Hanania *et al.*, 2010) and administration of inverse agonists (an agonist binding to a constitutively active receptor to stabilise it and reduce the activity) show positive signs in

murine models including decreased inflammation and mucous metaplasia (Lin *et al.*, 2008; Nguyen *et al.*, 2008). A further follow up study from the same group demonstrated that the same effect is achieved in β_2-AR null mice, indicating that it is the absence of constitutive signalling by the inverse agonists and not biased agonism causing the beneficial effects (Nguyen *et al.*, 2009).

Interestingly it has recently been shown that prolonged exposure of β_2-AR agonists can lead to a 50% decrease in SERCA2a protein in the heart (Ryall *et al.*, 2008). It is thought that this reduction in SERCA2a is due to a decline in gene transcription possibly via glycogen synthase kinase-3β (GSK-3β) which has been implicated as a negative regulator of SERCA2 gene transcription (Michael *et al.*, 2004). Ryall et al showed a 40% decrease in its phosphorylation upon β_2-AR stimulation thereby enabling more of the active form to directly interact with the promoter region of SERCA and inhibit its transcription.

Ojo (Thesis, University of London 2011) has shown that both formoterol and salbutamol decrease the expression of SERCA2 in healthy cultured ASM cells in a dose dependent fashion (see figure 1.9.2-1). The β_2-AR antagonist ICI-118551 inhibited the reduction suggesting the effect is via the receptor. Additionally stimulation with forskolin producing a similar rise of cAMP as formoterol also decreased SERCA2 expression indicating that it is a cAMP dependent effect. Finally pre-incubation with the ERK1/2 inhibitor U0126 blocked formoterol reducing SERCA indicating that ERK is involved in the signalling cascade. Under the same conditions the steroidal asthma treatment dexamethasone did not cause a decrease in SERCA. The next experiments now need to delineate the full intracellular signalling cascade from receptor activation to mRNA translation and whether this phenomenon occurs *in vivo*.



1.9.2-1 Changes in SERCA2 protein expression following stimulation with formoterol or forskolin for 24 hours, data values (mean ± SEM) represent SERCA2 expression levels expressed relative to untreated ASM cells, n=4-5 (Ojo, Thesis, University of London 2011).

1.9.3 Steroids

Corticosteroids form part of the first-line treatment for asthma and low doses control the symptoms well in many patients provided they adhere to a daily dosing regimen (Barnes, 2013a). Glucocorticoids diffuse into the cytoplasm where they can either: Bind with the glucocorticoid receptor alpha splice variant (GRα), homodimerise and then bind to glucocorticoid response elements (GRE) on steroid-responsive genes (Rhen *et al.*, 2005). The resulting complex either recruits co-activator or co-repressor proteins which can alter the structure of chromatin through acetylation and switch on anti-inflammatory genes or switch off inflammatory genes. Or they can interact directly with transcription factors such as NF-κB or with membrane associated proteins to impart ant-inflammatory actions in a non-genomic manner (Rhen *et al.*, 2005).

A small fraction of patients are resistant to corticosteroid treatment and they have been characterised by having a longer duration of symptoms, lower morning lung function and a greater degree of bronchial reactivity however these features are not exclusive to this patient subpopulation (Carmichael *et al.*, 1981). Several mechanisms have been identified which can lead to corticosteroid resistance including genetic susceptibility, defective GR binding and nuclear translocation, increased expression of the non-functional GR receptor splice variant GRβ, increased transcription factor activation or abnormal histone acetylation (Barnes, 2013a). Advances in understanding the molecular mechanisms behind steroid resistance have led

to new drugs being developed to restore sensitivity such as inhibitors of p38 mitogen-activated protein kinase (MAPK) (Bhavsar *et al.*, 2010) or antioxidants to enhance HDAC2 activity (Kirkham *et al.*, 2006). In a study looking at the function of the SR in aged rat myocardium it was found that dexamethasone improved SERCA function approximately 2-fold and decreased the time taken for relaxation (Narayanan *et al.*, 2004). A similar study has not been performed in ASM but if the finding translates then it would go further to explain the beneficial effects observed with the drug in asthma.

1.9.4 Anticholinergics

Anticholinergics or antagonists to the muscarinic acetylcholine receptor (mAChR) have long been used in asthma therapy due to their bronchodilatory actions inhibiting vagally induced smooth muscle constriction, historically taken by smoking deadly nightshade plant extracts containing atropine (Jackson, 2010). Acetylcholine production however may not be restricted to just parasympathetic nerve neuromuscular junctions but also from epithelial and immune cells (Wessler *et al.*, 2008). As such it is not surprising that muscarinic receptors are involved in more than just bronchoconstriction and have been implicated in airway remodelling and inflammation (Kistemaker *et al.*, 2012). It is thought that airway constriction via M_2AChR receptors releases TGF-β stored in the ECM via integrin interactions which enhances ASM remodelling (Oenema *et al.*, 2013b). Anti-inflammatory effects of anticholinergics have also been observed (primarily in COPD) and are thought to be attributed to their ability to reduce the release of chemotactic mediators from macrophages (Buhling *et al.*, 2007), limiting neutrophil chemotaxis (Vacca *et al.*, 2011) and reducing muscarinic dependent release of chemotactic factors from epithelial cells and ASM (Gosens *et al.*, 2009; Koyama *et al.*, 1998). The long acting antagonist tiotropium is used in asthma therapy and has been shown to be at least as effective at bronchodilation as β_2-AR (Gross, 1986) and could prove to be even more beneficial given the anti-inflammatory and remodelling effects.

1.9.5 PDE inhibitors/Xanthine derivatives

Theophylline, a phosphodiesterase (PDE)-4 inhibitor was originally used as a bronchodilator due to its cAMP raising effect (see section 1.3.2) and antagonising the adenosine receptor1 and 2. As β_2-AR agonists have come into use as first line therapy its use as a bronchodilator has declined and it is prescribed now due to the anti-inflammatory effects and HDAC2 activation to restore steroid sensitivity (Barnes, 2013b). The high expression of both PDE3 and PDE4 in leukocytes and their cAMP mediated inflammatory effects make them a useful target in inflammatory diseases. At low therapeutic doses theophylline reduces the late-phase asthmatic response as well as neutrophil and eosinophil numbers (Kraft *et al.*, 1996; Lim *et al.*, 2001; Sullivan *et al.*, 1994). The PDE4 mediated anti-inflammatory

mechanisms of theophylline are also responsible for many of its side effects which makes it a difficult target for drug research. Alternatives with improved safety profiles such as doxofylline (Shukla *et al.*, 2009), modified compounds to reduce side effects (Kawasaki *et al.*, 2014) or PI3 kinase inhibitors to improve HDAC2 activity offer more desirable solutions (To *et al.*, 2010). Dual inhibition of both PDE3 and PDE4 can offer positive interactions regarding both bronchodilation and anti-inflammatory effects to improve the therapeutic index (Abbott-Banner *et al.*, 2014). Still many have failed in the clinic however there are promising candidates such as RPL554 which is well tolerated due to its rapid plasma clearance and it elicits at least as effective bronchodilation as salbutamol with the addition of significant anti-inflammatory effects and mucociliary clearance in man (Franciosi *et al.*, 2013).

1.9.6 Anti-leukotrienes

Cysteinyl leukotrienes (cysLTs) are primarily synthesised in eosinophils and mast cells, they are derivatives of arachidonic acid metabolism by 5-lipoxygenase (5-LO) and exhibit both broncho-constriction and pro-inflammatory effects (Okunishi *et al.*, 2011; Scott *et al.*, 2013). There are two defined receptors for the Cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 which are $CysLT_1$ and $CysLT_2$. A third, as yet unidentified receptor has been postulated to be responsible for the inflammatory contribution of LTE_4 (Austen *et al.*, 2009). LTA_4, the precursor to the aforementioned cysLTs can also be hydrolysed to produce LTB_4, the principle leukotriene found in macrophages and neutrophils (Okunishi *et al.*, 2011). There are two targets within this class of drugs either antagonising the cysLT receptor-1 ($CysLT_1$) or inhibiting 5-LO. Only one study to date has compared the effectiveness between the two targets and the 5-LO inhibitor zileuton showed statistically significant greater improvements in both symptom scores and peak flow rate (Kubavat *et al.*, 2013). The result is possibly due to its less specific action of inhibiting the production of cysLTs rather than antagonising just $CysLT_1$ (Scott *et al.*, 2013). Anti-leukotrienes are not as effective at reducing inflammation as inhaled corticosteroids (Israel *et al.*, 2002) nor reducing bronchoconstriction as LABAs (Nelson *et al.*, 2000) therefore they are not part of the first-line of therapy in asthma. Their advantage lies however in greater patient adherence due to being taken as a once daily pill rather than an inhaler which has been shown to translate to equivalent effectiveness to steroids as a first-line therapy and LABAs as an add-on in a "real world" study (Price *et al.*, 2011; Scott *et al.*, 2013). Further rationalised research targeting the arachidonic acid derivative pathway could lead to improved therapies in this class of drugs especially in specific patient sub-populations such as neutrophil rich or exercise induced asthma.

1.10 Animal models

The low availability of asthmatic human tissue combined with the ethical considerations of performing invasive procedures on human subjects necessitates the use of animal models in asthma. When used in conjunction with *in vitro* and *ex vivo* techniques their usage can be reduced to a minimum while still gaining vital insights into whole-body physiological responses. The greatest understanding of the field can be made by drawing conclusions from a wide variety of approaches and integrating the results as shown when investigating contraction in ASM (Wright *et al.*, 2012a).

There are a plethora of different models available to study the various mechanisms thought to be driving the asthmatic phenotype however due to the disease's mixed aetiology none can fully replicate the human condition (Wanner, 1990). It is therefore wise to pick specific research questions you want answered about the disease and tailor the model specifically to answering that question. By doing this you can gain important insights into the various mechanisms one step at a time while minimising the compounding effects of each model's flaws.

A wide variety of animals have been used to study asthma, each with their own benefits and pitfalls. Non-human primates provide the closest resemblance to human physiology and models have been developed which closely match many markers of human asthma at a pathophysiological, cellular and molecular level (Ayanoglu *et al.*, 2011). In terms of the chronic natural disease only horses suffer from a spontaneously occurring pathology akin to asthma, heaves. However this may be more closely related to COPD due to the neutrophilic nature. It has been shown that horses with heaves have increased airway remodelling associated with an increased smooth muscle mass and myocytes proliferation (Herszberg *et al.*, 2006). The horse therefore is in a unique position to allow some direct cross-species comparisons to human asthma however the extent of the similarities still needs to be further defined. In light of ethical reasons it is better to use lower order animals such as rodents in place of primates.

It has been shown that guinea-pig lungs provide a more suitable model for human airway pharmacology than both rats and mice (Ressmeyer *et al.*, 2006). Along with rats and dogs but not mice they also show all three of the physiological changes in lung function following inhalation of allergens; early phase response, late phase response and airway hyperresponsiveness (Zosky *et al.*, 2007). A major drawback to all of these species however is their limited use in mechanistic models due to the low number of inbred strains and genetic knockouts, a trait where mice come into their own as research tools. A detailed knowledge of the mouse genome (Dietrich *et al.*, 1996) makes it easy to manipulate and produce transgenic strains.

Murine models of asthma involve sensitisation to an antigen followed by an exposure to that allergen to produce a well characterised T_H2 driven inflammatory response involving elevation of antigen specific IgE, eosinophilia and bronchial hyperresponsiveness (Zosky *et al.*, 2007). Ovalbumin has been the most widely used antigen historically but now more clinically relevant to human antigens are being used such as house dust mite (HDM) (Cates *et al.*, 2004). A secondary advantage to using a HDM exposure protocol is that an adjuvant such as aluminium hydroxide is not required to prime a T_H2 driven response. The ovalbumin model relies on T_H2 priming using an adjuvant (Brewer *et al.*, 1999) however this also serves to potentially distant the observed responses further from that seen in the clinical setting.

Different lengths of model also need to be taken into account depending what end points need to be evaluated. Acute models are good for examining AHR and the inflammatory infiltration to the airways however if remodelling is to be investigated a chronic exposure model is needed (Johnson *et al.*, 2004a; McMillan *et al.*, 2004). Remodelling involves altered ECM composition (Dolhnikoff *et al.*, 2009), subepithelial fibrosis (Tang *et al.*, 2006), enlargement of the ASM bundle (James *et al.*, 2012) and goblet cell hyperplasia (Rose *et al.*, 2006) as described in section 1.4. ASM hypertrophy and fibroblast accumulation have been negatively correlated with pre and post-bronchodilator FEV_1 values outlining the impact of remodelling on lung function (Benayoun *et al.*, 2003). T-cells and eosinophils can induce epithelial apoptosis via secretion of TNF-α and IFN-γ and necrosis via eosinophil cationic protein (ECP) (Trautmann *et al.*, 2002). Loss of the epithelial layer can result in reduced mucous clearance which coupled with enhanced goblet cell hyperplasia further occludes the airways. They also provide the initial barrier to spasmogens and can metabolise inflammatory mediators so their loss can further contribute to AHR by providing more direct access to nerve terminals (Bertrand *et al.*, 1993).

1.11 Summary

The following investigation centres on the findings that the intracellular handling of calcium ions in ASM cells derived from or induced by an asthmatic environment is altered (Jia *et al.*, 2013; Karner, 2010; Mahn *et al.*, 2009; Mahn *et al.*, 2010; Sathish *et al.*, 2012; Sathish *et al.*, 2009; White *et al.*, 2006). The recurring theme of these publications involves increased $[Ca^{2+}]_i$ either at rest, or following changes in ROCE or SOCE as a result of changes in the expression of critical calcium handling proteins on the SR and plasma membrane. Evidence shows that SERCA2 protein expression is reduced in a disease severity dependent manner in asthma (Mahn *et al.*, 2009) and that both TRPC3 and TRPC6 are altered by administration of cytokines implicated in asthma (Karner, 2010; White *et al.*, 2006). The work in this thesis furthers the current literature by exploring the impact of inducing an asthmatic phenotype (by stimulating with

cytokines) in healthy ASM cells on TRPC3, TRPC6 and SERCA2 expression, what these changes have on calcium handling and starting to unravel how these changes are mediated. By investigating the impact of the addition of inflammatory cytokines on healthy ASM and knocking down SERCA2 protein expression in naive mice, insights might be drawn over cause versus effect regarding the role of calcium homeostasis and the asthmatic phenotype.

The safety of β_2-AR agonists has been called into question (Rodrigo *et al.*, 2009) and there is evidence a decrease in SERCA2 expression may play a role (Ojo, 2011; Ryall *et al.*, 2008). The work presented here explores this phenomenon further to see whether changes in SERCA2 and subsequent calcium dynamics could explain the safety issues in the clinic.

An array of ASM cells derived from patients with differing asthmatic severities (healthy, mild and moderate) have been immortalised in Andrew Halayko's lab. The aim was to characterise them to ensure they retained their original phenotype during the immortalisation process as immortalised ASM cells are being used more commonly in this field without proper characterisation. The cells are immortalised by random insertion events of the human telomerase gene therefore the populations produced are heterogeneous. The large amount of cells present should provide a normalising effect on any detrimental insertions however there is a distinct possibility of phenotypes being lost or altered making characterisation essential.

The resulting changes in calcium transients observed experimentally could be indicative of changes in calcium oscillations which are key to encoding many of the phenotypes in ASM that are altered in asthma such as; enhanced proliferation, transcription and secretion of inflammatory mediators, contraction and migration (Mahn *et al.*, 2010). A better understanding of the processes and proteins involved in these changes could lead to the identification of new targets to prevent such changes and therefore the asthma pathogenesis.

1.12 Hypotheses

In this thesis I set out the following hypotheses:

1. That inflammatory mediators (TNF-α, TGF-β and IL-13) promote an asthmatic phenotype in ASM in part by altering the expression of key calcium handling proteins (SERCA2, TRPC3/6) leading to a dysregulation of calcium homeostasis.

2. That a murine *in vivo* model of reduced expression of SERCA2 will exhibit characteristics of asthma and these will be further enhanced after sensitisation and challenge with ovalbumin.
3. That the increased morbidity associated with chronic β_2-AR agonist use in asthma is due to a cAMP/PKA dependent reduction in SERCA2 expression.
4. That the immortalisation of ASM cells though random insertion of the hTERT gene will lead to retention of most but not necessarily all asthmatic disease specific phenotypes in the cell.

1.13 Aims

There were four broad aims of this thesis which are encompassed by each of the four results chapters:

1. The aim the first chapter was to discover the effects of the inflammatory cytokines TNF-α, IL-13 and TGF-β on the expression and function of calcium handling proteins in healthy ASM cells and how these are mediated.
2. The aim of the second chapter was to investigate whether the enhanced asthmatic phenotype observed *in vitro* when reducing SERCA2 protein expression is translated *in vivo* in a murine model of asthma.
3. The aim of the third chapter was to investigate how β_2-AR activation in ASM leads to SERCA2 down regulation and whether this occurs *in vivo*.
4. The aim of the final chapter was to characterise immortalised human ASM cells derived from a mixture of asthmatic disease severities to see if they retained their phenotypes.

Chapter 2 Materials and Methods

2.1 Isolation and culture of human ASM cells

ASM cells were obtained from patients in accordance with procedures approved by the South East London Research Ethics Committee by deep endobronchial biopsy, REC reference number: 10/H0804/66. Informed written consent was obtained from the patients prior to the procedure. Biopsies were excised from the right middle and lower bronchi using Olympus cupped forceps (model FB-35-C1).

2.1.1 Patient criteria:

All: Aged 18-70 years.

Asthmatics: History of typical symptoms over at least two years with a minimum 12% reversibility and/or a methacholine bronchial challenge of <8mg.ml^{-1}. All asthmatics used to generate the immortalised cell lines were deemed "moderate" in severity as their predicted FEV_1 values fell between 70-90%.

Healthy: Life-long absence of symptoms, and lung functions within normal limits.

Exclusion criteria: Patients on leukotriene receptor antagonists, current smokers, pregnant/lactating females or past/present disease deemed to affect outcome of study.

2.1.2 ASM dissection

ASM bundles were isolated by dissecting away the surrounding tissue under a microscope in HBSS in DMEM containing 10% FBS, L-glutamine (2mM), sodium pyruvate (1mM), non-essential amino acids and fungizone (2µg.ml-1) (referred henceforth as DMEM + all). They are then cleaned with HEPES (no Ca^{2+}, no EGTA and 1g/L glucose) solution twice and digested using the following enzymatic solution:

In 2ml HEPES (no Ca^{2+}, no EGTA and 1g/L glucose).

- 4mg colagenase Type XI (0.2% w/v)
- 2mg papaine (0.1% w/v)
- 2mg trypsin inhibitor (0.1% w/v)
- 20µl DTT 100mM stock (1mM)

HEPES solution was removed and the tissue incubated in the enzymatic mix for 30 minutes at 37°C, inverting every 5 minutes 6-8 times. The cells were then washed three times in warm HEPES by spinning the cells down at 100*g*, aspirating and resuspending. A further three washes were then carried out in the

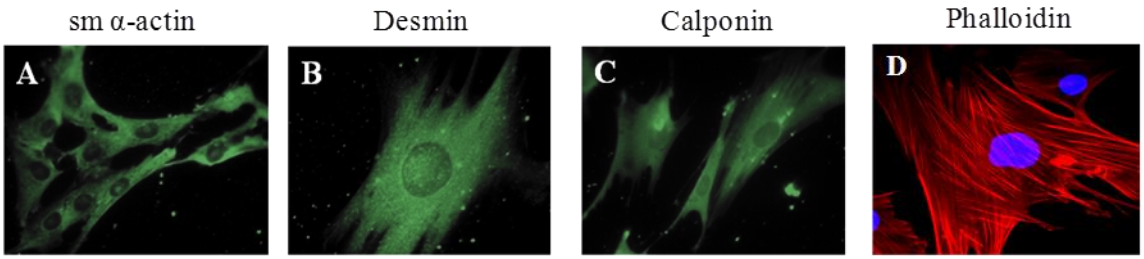
same way using DMEM + all media. Cells were dispersed using a flame polished glass pipette into a T12.5 or T25 culture flask containing DMEM + all media depending on the size of the pellet. Cells were then cultured at 37°C and 5% CO_2 replacing the media after 24 hours. Depending on the quality of the biopsy the cells were ready for their first passage in 10-14 days.

2.1.3 ASM phenotype confirmation

To confirm the cells grown in culture are ASM they were stained at an early passage soon after P0 and again at later passages to show they haven't differentiated too far from their explanted state. The cells were confirmed as ASM cells via immunocytochemistry staining using specific markers; calponin, desmin and α-actin (Halayko *et al.*, 1996). Calponin is a thin filament protein that regulates smooth muscle contraction, desmin is an intermediate filamentous protein found in smooth muscle and smooth muscle α-actin stains for cytoskeletal actin filaments in smooth muscle cells. Depending on the treatment of the cells, such as to what level of confluency they were allowed to reach before splitting and whether or not they are serum starved these (and other) markers will appear in varying degrees (Wright *et al.*, 2012b).

ASM cells were grown on 13mm diameter cover slips to approximately 30-50% confluency and fixed using 4% (w/v) paraformaldehyde and 4% (w/v) PEG6000 in PBS-ME (3mM EGTA, 2mM $MgCl_2$) at room temperature for 30 minutes. The cells were then washed twice in cold PBS-ME and permeabilised using 0.1% Triton X-100 for 1 minute, washed a further two times and then blocked with 5% filtered BSA (w/v) in PBS-ME at room temperature for 1 hour. After a further two washes primary antibody at a 1 in 100 dilution in 1% filtered BSA was added at 4°C overnight. The cover slips were again washed twice with a further two, 5 minute washes before adding the secondary Alexafluor secondary antibody at a 1 in 300 dilution for 2-3 hours at room temperature in the dark to prevent photobleaching. The cells were washed twice again with the further two 5 minute washes and incubated for 20 minutes at room temperature with phalloidin:TRITC (a toxin which binds to F-actin, thus revealing the cytoskeletal arrangement of the cell). After repeating the previous wash step cells were incubated with Hoechst33342 (Life Technologies™) for 10 seconds, washed again and mounted onto glass slides using Mowiol Mounting Media (10% w/v Mowiol 4-88 and 25% v/v glycerol in 0.1M Tris) and left to set overnight at RT in a dry dark place. Mowiol 4-88 is a polyvinyl alcohol which prevents bleaching of the fluorescent probes. The Hoechst33342 counter-stains the nuclei by binding to double stranded DNA and emitting a blue fluorescent light around 461nm.

All the cells used in the following experiments stained positive for all three markers, an example is shown in Fig. 2.1.3-1 below:



2.1.3-1 Staining for ASM cell markers; sm-α actin (A), desmin (B), calponin (C) and phalloidin (D). Picture D has been counter stained to show the nuclei using Hoechst stain. Cells in pictures are derived from the healthy patients.

2.2 Cell culture medium

Different recipes of cell culture medium were used during isolation, culture and experiments depending on the requirements of the cells. Hank's Balanced Salt solution (HBSS) containing gentamycin (Gibco) and amphotericin B (Thermo Scientific) were used to collect the biopsies from the patients.

During the maintenance of cell lines they were allowed to grow to approximately 80% confluency before splitting for experiments or into a new flask. The media used for during this phase is Dulbecco's Modified Eagle Medium (DMEM) containing 1g/L glucose, 10% FBS, L-glutamine (2mM), sodium pyruvate (1mM), non-essential amino acids, gentamycin (50µg/ml) and amphotericin B (2µg/ml). Splitting was performed by removing all of the culture media, washing the flask with warm sterile phosphate buffered saline (PBS), adding a small volume of 0.025% trypsin/EDTA (ethylenediaminetetraacetic acid) and incubating for 3-5 minutes at 37°C. The trypsin was subsequently neutralised by adding a surplus of culture media (containing serum) and the cell suspension was centrifuged at 200*g* for 5 minutes. The supernatant was removed and the cell pellet is resuspended in an appropriate amount of media to continue the cell line at a desired cell density in the flask of choice.

Prior to each experiment cells were growth arrested for 72 hours to ensure they are all at the same stage of the cell cycle and of a uniform phenotype when the experiment begins. The media used to achieve this (serum free media) contained BSA (1%), transferin (5µg/ml), insulin (1µM), ascorbate (100µM) and all of the contents above in the maintenance media with the exception of FBS.

2.3 Protein isolation and measurement

ASM cells were seeded in culture flasks and grown to approximately 80% confluency and growth arrested for 72 hours prior to the start of the experiment in serum free media. Cells were then subjected to various experimental conditions for the specified length of time at 37°C 5% CO_2, 95% air.

To terminate the experiment culture dishes were placed on ice and the media is swapped for ice cold PBS to wash and slow any further reactions. The cells were then lysed using 1x lysis buffer (Cell Signaling) containing 1x protease inhibitor (Sigma) at room temperature for 10 minutes with regular vortexing, before returning to ice. Samples were centrifuged at 10,000*g* for 3 minutes and the supernatant stored at -80°C.

2.3.1 Total protein assay

To measure the protein concentration in each sample to allow equal protein loading in each lane of the western blot, the Bichinchoic Acid (BCA) assay was used (Pierce) (Smith *et al.*, 1985). A serial dilution of bovine serum albumin with a top concentration of 2mg.ml^{-1} was used as the standard curve to compare the unknown samples against and the samples are diluted down to fall within that range. The assay relies on the biuret reaction which is the reduction of copper from Cu^{2+} to Cu^{1+} in the presence of protein. The BCA solution containing 4% copper sulphate was added for 30 minutes at 37°C to give a purple chromophore with a maximum absorbance of 574nm which can be read on a spectrophometer (Anthos HT II Life Technologies™, UK). The yield of the purple chromophore is linearly related to the amount of protein present and using the standard curve the graph can be interpolated to calculate the concentration of the unknown samples.

2.3.2 Western blotting

Western immunoblots were used to compare relative protein expression levels between the protein of interest and a control protein unaffected by the experiment. 10-40µg of protein lysates were run through a 4-12% Bis-Tris gel (Life Technologies™) at 180V for approximately one hour. Sodium dodecyl sulphate polyacrylamide gel electrophoresis separates proteins according to their molecular weight by disrupting their secondary structure and adding an even negative charge across the polypeptide chain. Thus larger proteins carry a stronger charge. Once the gel has run and the samples have separated they are subsequently transferred onto a nitrocellulose membrane using the Life Technologies™ X Cell II Blot Module and ran at 30V for one hour. Membranes were then blocked in 5% milk in TBS Tween-20 for one hour at room temperature. Primary and secondary antibodies were also prepared in 5% milk in TBS Tween-20 with the primary antibody incubation lasting approximately 18 hours at 4°C and the HRP conjugated secondary antibody for 1-2 hours at room temperature. Membranes were washed in TBS Tween-20 in between. An enhanced chemiluminescence (ECL) agent, commonly either ECL-Plus or Femto ECL (GE Healthcare Life Technologies™ and Pierce respectively) were used as the horseradish peroxidase substrate depending on the amount of protein of interest present. Autoradiography film (Kodak) was exposed until the bands emerged and subsequently developed. Analysis was performed using ImageJ v.1.44p.

The film and developer technique was later upgraded to using the Bio-Rad ChemiDoc MP system where the chemiluminescence is measured directly by a cooled-charge-coupled device camera, vastly improving the dynamic range and sensitivity.

2.3.3 ELISA

Enzyme linked immunosorbant assays are used to quantify the amount of a particular protein within a sample. Briefly, a primary antibody directed against the protein of interest was added to a 96 well plate and incubated at room temperature overnight. Bovine serum albumin (BSA) was added to block unspecific binding, and then sample and the standards provided were added to the appropriate wells. The samples had to be diluted in reagent diluent (BSA) 1:5 so that they fell within the standard curve range. A biotinylated secondary antibody directed against the protein of interest binds to the sample/primary antibody complex. Horseradish peroxidase (HRP) conjugated to streptavidin was then added which binds to the biotin on the secondary antibody. A soluble substrate was then added which changes colour over time, a reaction which is dependent upon the amount of HRP present. When the desired colour change has occurred the reaction was stopped by adding sulphuric acid and the plate is measured at 450nm minus the reading at 570nm to correct for optical imperfections in the plate. Between steps excess unbound samples were washed off using 0.05% Tween 20 in PBS. The detection limits of the ELISAs can be found below.

Assay Type	**Biomarker**	**Detection limits**
BCA Total Protein	Total protein	0.1-2.0 $mg.ml^{-1}$
DuoSet ELISA	Eotaxin-1	15.6-2000 $pg.ml^{-1}$

2.4 Calcium signalling

2.4.1 Using the plate reader

Calcium responses to bradykinin were measured using a GloMax®-Multi Detection System plate reader (Promega, Madison, USA). Briefly, cells were cultured in 48-well plates and serum starved for 72 hours. Serum was removed and each well washed with PBS (esterase activity in the culture media provides a source of baseline fluorescence) which in turn was removed and Fluo-4NW dye and probenecid added. The final concentration of 2.5mM probenecid inhibits the extrusion of dye from the cells via anion transporters. Materials and instructions were supplied as the Fluo-4 NW Calcium Assay Kit (Life Technologies™).

The plates were incubated at 37°C for 40 minutes, followed by room temperature for 40 minutes and then read using the following protocol:

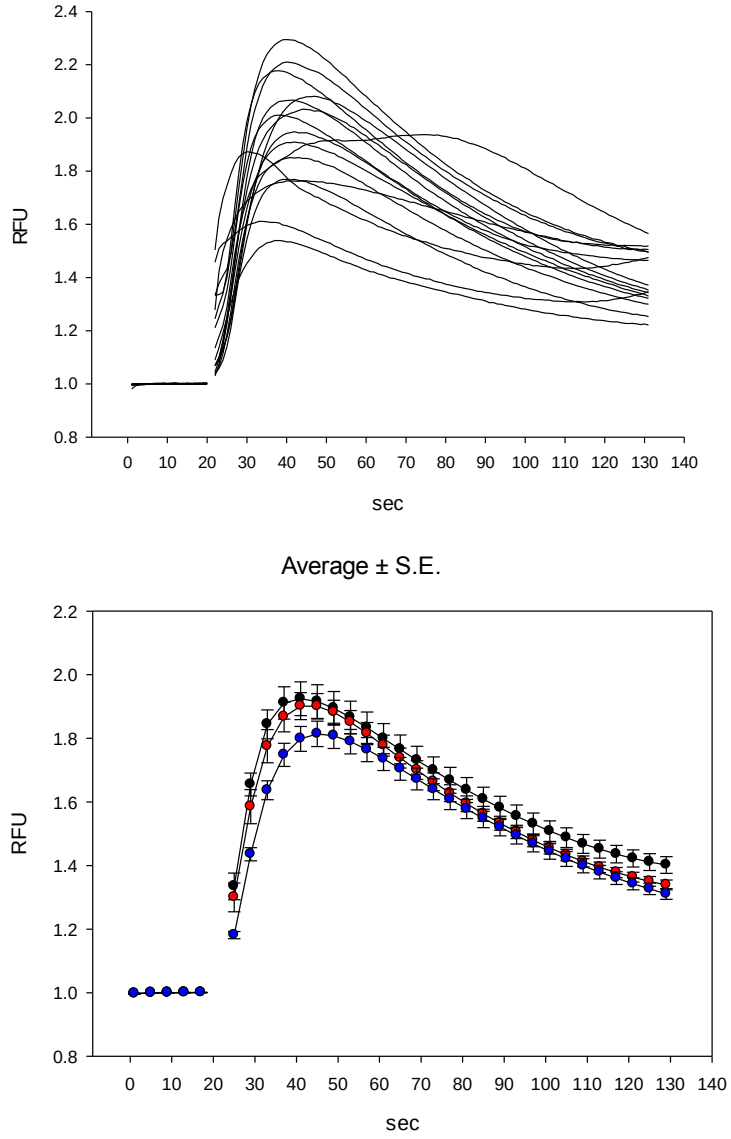
For each well; Baseline recording - 0 sec delay

20x readings

	1 sec interval
Calcium transient -	25µl injection 0.5µM (final concentration) bradykinin
	0.5 sec delay
	180x readings
	1 sec interval

Excitation wavelength is 490nm and emission wavelength is 510-570nm.

The technique was trialled on several lines and treatments and although it was relatively high throughput as 48 well plates could be read in a couple of hours the data was extremely variable within each condition. Fig. 2.4.1-1 (top) shows the high variation generated by stimulating identically treated control cells with 0.5µM bradykinin. Such a high variability reduced the power of the experiments to such a degree that differences between treatments observed previously using the microspectrofluorimeter were no longer observed (Fig. 2.4.1-1 bottom).



2.4.1-1 Calcium transients generated using the GloMax®-Multi Detection System in response to 0.5µM bradykinin. Top: Unstimulated control data from 16 replicates shows very high variability. Bottom: Comparing unstimulated (black), 10ng.ml^{-1} TGF-β (red) for one week and IL-13 and TGF-β both 10ng.ml^{-1} for one week. Points represent mean ± SEM.

2.4.2 Using microspectrofluorimeter

Cells were grown to 80% confluency on 13mm cover slips and serum starved for 72 hours as previously described. The cover slips were loaded for 45 minutes with 1µM Fura PE 3-AM (Sigma) and mounted onto a RC-25 perfusion chamber (Warner Instrument Co) and perfused with HEPES buffer comprised of; 130mM NaCl, 4mM KCl, 1mM $MgCl_2$, 2mM CaCl, 5.5mM D-glucose and 10mM HEPES. When the dye is inside of the cell cytoplasmic esterases hydrolyse the membrane permeant AM ester derivatives trapping the indicator in the cytosol (Vorndran *et al.*, 1995). Fura-PE3 undergoes a shift in its peak absorption

wavelength of 380nm to 340nm when binding to calcium while the peak emission wavelength is constant at 510nm. The chamber was mounted on an inverted epi-fluorescence microscope (Nickon ECLIPSE TE2000) with a x20 UV objective lens. A 75W Xenon arc light source (Cairn Optosource) was used to excite the dye and a computer controlled monochromator (Cairn Optoscan monochromator 2.11) switched the wavelengths between 340nm and 380nm with 15ms dwelling time. The emission signal was detected by a photomultiplier detector and changes in intracellular calcium ions measured as the ratio of emission intensities at >510nm caused by 340nm and 380nm excitation respectively. Twenty-five data points were collected each second and averaged to produce 1 value per second using the Cairn Research Acquisition Engine Software v.1.7.

Once a steady baseline had been achieved, 100 seconds of control readings were taken followed by 200 seconds of perfusion with the agonist. If a subsequent agonist was to be used then a 400 second washout period was given in between. Measurements were taken as the difference between the peak response and the baseline reading immediately before. The decay rates were calculated by fitting the data points from the peak to baseline with a single, 3 parameter exponential decay fit (Mahn *et al.*, 2009).

2.4.3 Flufenamic acid

Flufenamic acid (FFA) was originally used as a non-steroidal anti-inflammatory drug due to its inhibition of cyclo-oxygenases however its use dwindled due to poor benefits compared to its numerous drawbacks. However, it has been continually used to this day in the lab because it regulates many ionic currents within the cell (Guinamard *et al.*, 2013). Although this opens up its use to study numerous channels it presents a large issue when it comes to interpreting data obtained and therefore any conclusions must be treated with caution. On top of the broad range of targets FFA can affect, results on individual channels are inconsistent between publications adding further importance on considered deductions from results (Inoue *et al.*, 2001; Klose *et al.*, 2011). Flufenamic acid has been shown to act as a TRPC6 agonist (Foster *et al.*, 2009) justifying its use here.

There is a dearth of specific agonists and antagonists which can be used as pharmacological tools to investigate TRPC channels so when using a compound such as FFA appropriate controls must be used for a useful interpretation of the data.

2.4.4 Store operated calcium entry

Store operated calcium entry (SOCE), formerly known as capacitative calcium entry (CCE) is the process by which intracellular calcium stores are replenished via influx from outside the cell. To make this

measurement cells were perfused with HBSS solution containing no calcium, the stores were then emptied and reuptake prevented by stimulating with a combination of 1µM ionomycin (Morgan *et al.*, 1994) to empty the stores and 1µM thapsigargin to block SERCA2 activity. Finally calcium was reintroduced into the perfusate and as it entered the cytoplasm the change in 340nm to 380nm ratio from fura-PE3 excitation was measured.

2.5 Measuring cell proliferation

Two methods have been used to measure ASM cell proliferation, the MTT assay and the tritiated thymidine incorporation assay. Each requires ASM cells seeded at $9x10^3$ cells per well in a 96 well plate, grown for 24 hours in full serum containing media then washed with sterile PBS and serum starved for 72 hours.

2.5.1 MTT assay

The yellow tetrazolium salt 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) is reduced by metabolically active cells by NADPH. The resultant intracellular purple formazan can be solubilised and quantified by spectroscopic means at a wavelength of 574nm. After serum starvation the cells were incubated with increasing concentrations of FBS (0, 2.5, 5 or 10%) and grown for 48 hours before the media was removed and washed with PBS. 100µl of HBSS containing 2mM calcium and $1g.L^{-1}$ glucose was added to each well with 10µl of 12mM MTT and incubated at 37°C for 4 hours. 1g of SDS was added to 10ml of 0.01M HCl, 100µl of this solution was then added to each well and incubated for a further 4 hours at 37°C and read at 574nm. Even though this is strictly a measure of metabolic activity and not directly cellular proliferation it has been shown to correlate very well with increases in ASM cell number following a graded stimulation with FBS (Hirst *et al.*, 1992).

2.5.2 ^{3}H-thymidine incorporation

Following serum starvation the cells were incubated with increasing concentrations of FBS, akin to the MTT assay for 4 hours and then pulsed with 3.7 Bq of ^{3}H-thymidine (Amersham). The cells were then left to grow and incorporate the thymidine for 48 hours before being lysed via freezing at -20°C for storage. The lysates were then vacuumed onto filter paper and mixed with a scintillation fluid and counted on a beta-counter (TopCount NXT, PerkinElmer) as described previously (Mahn *et al.*, 2009).

2.6 shRNA as an experimental tool

Introduction of double stranded RNA (dsRNA) to a cell results in RNA interference and suppression of protein production, termed post transcriptional modification. An expression vector containing the

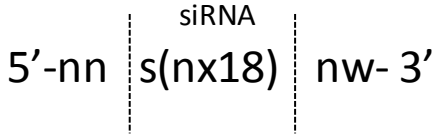
sequence for the short hairpin RNA (shRNA) was introduced into the cell by electroporation (see 2.6.5) where upon it translocates to the nucleus and is transcribed by RNA polymerase. The primary transcript contains a hairpin loop which is processed by Drosha and DGCR8 (Lee *et al.*, 2003) to produce shRNAs with a 2 base pair 3' overhang (Zhang *et al.*, 2002). The shRNA is then transported into the cytoplasm where it forms a complex with the RNase-III-family enzyme called Dicer in an ATP dependent process to generate short interfering siRNA (siRNA) by cleaving the hairpin. The siRNA is incorporated into a RNA induced silencing complex called RISC where the two strands of the siRNA are separated leaving the single complementary strand available to perform RNA interference.

When using as an experimental tool a control siRNA which has no homology to mRNA must be used to normalise any protein expression changes occurring due to the transfection method. The protocol was used as described previously in the lab (Knock *et al.*, 2008).

2.6.1 Design and synthesis

RNA interference is a very useful tool employed to discover the function of certain proteins within a cell signalling cascade.

To analyse the role of β-arrestin-2 in the down-regulation of SERCA2 following stimulation of ASM cells with salmeterol and the role of Smad2 and 3 in the regulation of various protein expression following TGF-β stimulation, a pool of siRNAs were made to target them. If the target protein has multiple splice variants a section of the sequence present in all of them was used to design the siRNA using the algorithm: **n2sn19w** n=A/T/G/C, s=G/C, w=A/T designed and optimised by Dr Yasin Shaifta. From the results generated a pool of three were selected which contain the highest content of A/T in the final 3' seven positions of the siRNA.



The 19 nucleotide target sequences were compared with the human genome database using BLAST (www.ncbi.nlm.nih.gov/BLAST) and any sequence showing ≥15 bp homology to an off-target protein was omitted from consideration. 5' single-stranded overhangs (BamHI and HindIII) were designed on the end

of each 64-65[1] mer insert as well as a 9-nucleotide spacer between the sense and antisense sequences (TTCAAGAGA, italics in Table 2.6-1). A poly-T tail was added immediately prior to the 3' restriction end of the sense oligonucleotide to serve as an RNA polymerase III termination site. The oligonucleotides were synthesised by Sigma and resuspended in ddH_2O to a stock concentration of 100µM. The sequence of all the oligonucleotides designed and their targets are in Table 2.6-1.

[1] An additional guanine is added immediately 5' to the start of the siRNA sequence on the sense strand if the first base of the siRNA is T or C (underlined in Table 2.6-1). The purpose of this is to increase the efficiency of RNA polymerase III transcription.

Table 2.6-1 siRNA oligonucleotides

mRNA	Accession number	Hairpin oligonucleotide (target sequence in bold)	
B-Arrestin-2	NM_004313	F1	5'-GATCCCG**CTGTGCTAAATCACTAGAA***TTCAAGAGA*TTCTAGTGATTTAGCACAGTTTTTTGGAAA-3'
		R1	5'-AGCTTTTCCAAAAAA**CTGTGCTAAATCACTAGAA**TCTCTTGAATTCTAGTGATTTAGCACAGCGG-3'
		F2	5'-GATCCC**GACCGTCAAGAAGATCAAA***TTCAAGAGA*TTTGATCTTCTTGACGGTCTTTTTTGGAAA-3'
		R2	5'AGCTTTTCCAAAAAA**GACCGTCAAGAAGATCAAA**TCTCTTGAATTTGATCTTCTTGACGGTCGG-3'
		F3	5'-GATCCCG**CCAACCTCATTGAATTTGA***TTCAAGAGA*TCAAATTCAATGAGGTTGGTTTTTTGGAAA-3'
		R3	5'-AGCTTTTCCAAAAAA**CCAACCTCATTGAATTTGA**TCTCTTGAATCAAATTCAATGAGGTTGGCGG-3'
Smad2	NM_005901	F1	5'-GATCCC**GATTGCCACATGTTATATA***TTCAAGAGA*TATATAACATGTGGCAATCTTTTTTGGAAA-3'
		R1	5'-AGCTTTTCCAAAAAA**GATTGCCACATGTTATATA**TCTCTTGAATATATAACATGTGGCAATCGG-3'
		F2	5'-GATCCC**GAGCCACAGAGTAATTATA***TTCAAGAGA*TATAATTACTCTGTGGCTCTTTTTTGGAAA-3'
		R2	5'-AGCTTTTCCAAAAAA**GAGCCACAGAGTAATTATA**TCTCTTGAATATAATTACTCTGTGGCTCGG-3'
		F3	5'-GATCCC**GTGTTCGATAGCATATTAT***TTCAAGAGA*ATAATATGCTATCGAACACTTTTTTGGAAA-3'
		R3	5'-AGCTTTTCCAAAAAA**GTGTTCGATAGCATATTAT**TCTCTTGAAATAATATGCTATCGAACACGG-3'
		F4	5'-GATCCCG**CCACCTCCTGGATATATCA***TTCAAGAGA*TGATATATCCAGGAGGTGGTTTTTTGGAAA-3'
		R4	5'-AGCTTTTCCAAAAAA**CCACCTCCTGGATATATCA**TCTCTTGAATGATATATCCAGGAGGTGGCGG-3'
		F5	5'-GATCCCG**CGTCTATCAGCTAACTAGA***TTCAAGAGA*TCTAGTTAGCTGATAGACGTTTTTTGGAAA-3'
		R5	5'-AGCTTTTCCAAAAAA**CGTCTATCAGCTAACTAGA**TCTCTTGAATCTAGTTAGCTGATAGACGCGG-3'
		F6	5'-GATCCC**GAATGTGCACCATAAGAAT***TTCAAGAGA*ATTCTTATGGTGCACATTCTTTTTTGGAAA-3'
		R6	5'-AGCTTTTCCAAAAAA**GAATGTGCACCATAAGAAT**TCTCTTGAAATTCTTATGGTGCACATTCGG-3'
Smad3	NM_005902	F1	5'-GATCCC**GTGAGTTCGCCTTCAATAT***TTCAAGAGA*ATATTGAAGGCGAACTCACTTTTTTGGAAA-3'
		R1	5'-AGCTTTTCCAAAAAA**GTGAGTTCGCCTTCAATAT**TCTCTTGAAATATTGAAGGCGAACTCACGG-3'
		F2	5'-GATCCCG**CCCAGCACATAATAACTTG***TTCAAGAGA*CAAGTTATTATGTGCTGGGTTTTTTGGAAA-3'
		R2	5'-AGCTTTTCCAAAAAA**CCCAGCACATAATAACTTG**TCTCTTGAACAAGTTATTATGTGCTGGGCGG-3'
		F3	5'-GATCCC**GCAACCTGAAGATCTTCAA***TTCAAGAGA*TTGAAGATCTTCAGGTTGCTTTTTTGGAAA-3'
		R3	5'-AGCTTTTCCAAAAAA**GCAACCTGAAGATCTTCAA**TCTCTTGAATTGAAGATCTTCAGGTTGCGG-3'

siRNA	Sequence
Adrenergic b-2 receptor (s1123) siRNA	AAUGUUAACGAUGAAGAAGgg

2.6.2 Annealing the oligonucleotides

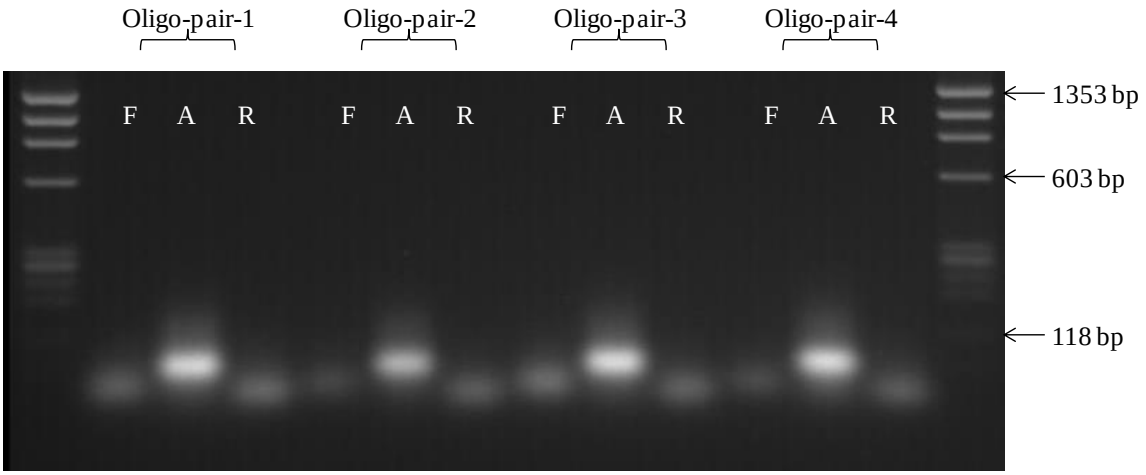
The two complementary oligonucleotides were annealed together by adding 1µl of both the forward and reverse sequences (100µM stock), 50mM NaCl and 16µl ddH_2O in the following 20µl reaction:

95°C for 20mins

90°C for 10mins

Temperature was reduced in 5°C increments down to 20°C and held at each temperature step for 10 minutes and finished at 4°C.

Then 2µl of stock single forward and reverse oligonucleotides was denatured at 95°C for 5 minutes with 50mM NaCl and 16µl ddH_2O in a 20µl reaction. They were then loaded either side of the annealed oligonucleotides on a 2.5% agarose gel with the φx174 DNA/BsuRI (Hae III) marker 9 (Fermentas). The results showed all of the annealing was successful, an example blot is shown below.



2.6.2-1 The annealed products of four oligonucleotide pairs all showing successful annealing. Forward (F), annealed (A) and reverse (R) siRNA oligonucleotides were run side by side.

2.6.3 Ligation and transformation

Ligation was achieved by mixing 270ng of the plasmid vector (pSilencer 3.0-H1, Life Technologies™), 15µM of the annealed oligonucleotides, 1x T4 buffer, 2 Weiss units of T4 ligase and made up to 10µl with ddH_2O and left overnight at 16°C.

Transforming One Shot® TOP10 *E. Coli* bacteria (Life Technologies™) with the plasmid ensures stable replication of high copy number plasmids. 2µl of the ligated plasmid was added to 25µl of bacteria and

incubated on ice for 30 minutes. The bacteria was then heat shocked for 30 seconds at 42°C in a water bath and returned to ice for a further 2 minutes. 125µl of Super Optimal broth with catabolite repression (SOC) was added and shaken at 37°C for 1 hour. 50µL was spread onto warm LB/agar plates containing 100µg/mL ampicillin and colonies were then grown overnight at 37°C.

To make LB broth and agar:

For 2 litres: 20g Tryptone - pancreatic digest of casein (Becton and Dickinson)
10g Yeast extract (Becton and Dickinson)
20g NaCl
2L ddH_2O

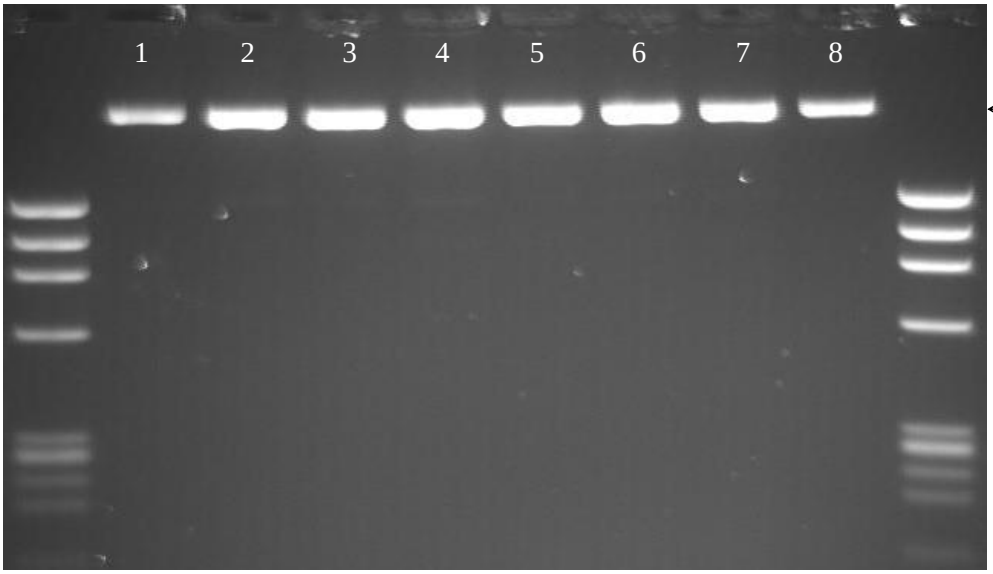
For agar: 7.5g agar (Becton and Dickinson)
500ml LB

Both mixtures were autoclaved to sterilise and dissolve the agar solution.

Four colonies from each of the three siRNAs in the pool were picked and grown in 5ml LB broth and ampicillin overnight at 37°C and purified using the QIAgen Miniprep kit (#27104, QIAgen).

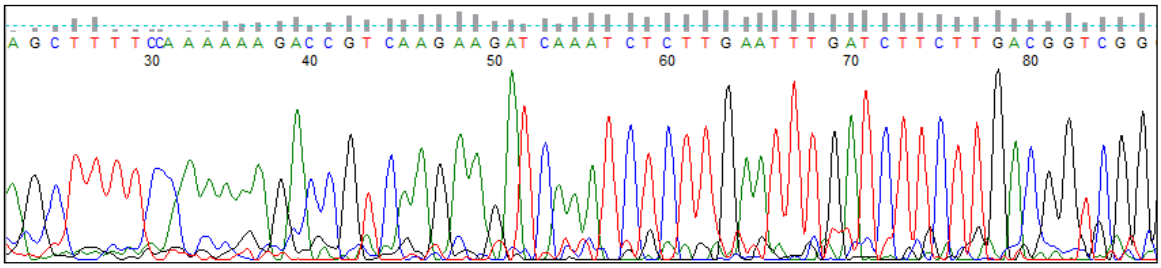
2.6.4 Verification of insert by digestion and sequencing

The vector contains the H1 polymerase III promoter capable of generating large amounts of shRNA. The plasmids contained two rare EcoR1 sites, one just before the H1 promoter region and a second between the BamHI and HindIII restriction sites. Following excision of this region and re-ligation with the new cloned shRNA sequences an EcoRI digestion was performed as a quality control check. If the new insertion was complete only a single fragment would be produced from the linearised product of 2795bp, however if the original insert was still present two fragments would appear on the gel of 122bp and 2653bp. Fig. 2.6.4-1 shows that all ligations were successful.



2.6.4-1 EcoRI digest of eight SMAD2 siRNA mini preps. Lanes 1-4 are from the second pool created and lanes 5-8 from the third. All ligations shown were successful.

All of the samples were then sequenced using the Reverse (3.0rev) primer (5'-GAGTTAGCTCACTCATTAGGC-3') and only the colonies showing successful cloning were tested for knockdown efficiency as shown in the relevant results section.



2.6.4-2 Sequencing result from the second β-arrestin-2 siRNA. When compared with R2 from Table 2.6-1 there are no differences demonstrating the cloning was a success.

2.6.5 Transfection of ASM cells

The ASM cells were transfected using electroporation by the AMAXA Nucleofector® kit supplied by LONZA. The kit has numerous pre-set programmes with varying electrical waveforms to transfect primary ASM cells while minimising cell death, the programme used in this study was A-33. 100µl of their Nucleofector® resuspension solution (82µl basic Nucleofector® solution and 18µl of supplement) was added to 0.5-2x10^6 cells pelleted along with either the scrambled siRNA or the on target siRNA. The cells were then transfected and resuspended in culture medium to neutralise the toxicity of the resuspension

medium before being seeded in cell culture. While typically between 20-30% cell death occurs, dead cells do not adhere and are washed off the following day. Efficiency of the knockdown was confirmed by probing for the protein via western blot (see section 2.3.2).

2.7 Nucleic acid

2.7.1 RNA extraction and purification

RNA was extracted from ASM cells following treatment using the QIAgen RNeasy® Mini-kit. RNase free consumables were used in addition to cleaning work surfaces with RNase free disinfectant to prevent loss of RNA.

Cells were washed with PBS, lysed with beta mercaptoethanol and buffer RLT and purified by following the QIAgen RNeasy® kit. DNA was removed using Turbo DNase (Life Technologies™) one unit per 20µg RNA, incubated at 37°C for an hour thus ensuring no trace DNA is amplified during the PCR reactions.

Purified sample was quantified using the NanoDrop 1000 spectrophotometer (Thermofisher) to determine nucleic acid concentration at 260nm and impurities such as protein at 280nm. Samples were deemed of an adequate purity with a 260nm to 280nm ratio between 1.8-2.1.

2.7.2 Reverse transcription

Quantitative polymerase chain reactions (qPCR) require double stranded DNA, therefore the single stranded RNA must be converted to cDNA. 2µg of RNA was added to 400ng of random hexamer mix and incubated at 70°C for 5 minutes and left on ice. 10mM dNTPs were then added with the suitable buffer and ddH_2O and incubated at 25°C for 5 minutes. 400 units of RevertAid H Minus M-MuLV Reverse Transcriptase was added and incubated at 25°C for 10 minutes, then at 42°C for 60 minutes and finally the reaction was stopped at 70°C for 10 minutes to inactivate the enzyme and chilled on ice. Samples were then ready for PCR.

2.7.3 Quantitative PCR

TaqMan qPCR tells you the relative quantity of mRNA transcribed from a particular gene of interest compared to a house keeping gene which is assumed to be unaltered by the experimental conditions. Two primers are designed against exon sequences from the gene of interest and a probe containing a fluorescent dye on one end and a quencher on the other. The accumulation of fluorescence from the reporter dye (FAM) increases with each PCR cycle in a sigmoidal fashion. As the Taq polymerase extends the primer to replicate the cDNA from 3' to 5' its exonuclease activity from 5' to 3' degrades the sequence specific probe

annealed to the cDNA of interest separating the fluorophore from the quencher thus creating a fluorescent signal. A threshold level of fluorescence in the linear portion of the curve is applied and the amount of cycles taken to reach this is recorded. As the amount of starting material is proportional to the cycles required to reach the threshold level of fluorescence expression of the gene of interest can be quantified. The reference housekeeping RNA used in these studies was 18S rRNA tagged to a different reporter dye (VIC).

In a 384 well plate a mixture of 10µM forward and reverse primers (6µM for 18S), 2x PCR master mix (Applied Biosystems), 100ng cDNA were adjusted to a final volume of 15µl with RNase free water and run in triplicate. The reaction was initiated at 50°C for 2 minutes and raised to 95°C for 10 minutes before 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. Results were recorded on Applied Biosystems ViiA 7 Real-Time PCR System and quantified using ViiA™ 7 Software (Applied Biosystems). ΔCt was obtained by subtracting 18S average Ct values (cycle threshold) from the average Ct values from the gene of interest. All values in the results sections are given as relative quantities (RQ) which is the dependent experimental variable ΔCT value normalised to its relevant control ΔCT value.

2.8 Myography

Ex vivo contraction measurements on isolated bronchioles were carried out using a Mulvany-Halpern type small vessel wire myograph (DMT, Denmark). It comprises of two metal jaws, one of which is attached to a force transducer, the other to a micrometer arm which can be adjusted manually to stretch the airway. It is important when making isometric force measurements to start with tension in the airway walls to prevent changes in lumen diameter. Force production and sensitivity to agonists are also dependent on muscle stretch so comparable levels must be maintained between treatment groups.

Bronchioles were incubated at 37°C in Krebs-Henseleit solution, a physiological salt solution containing 118mM NaCl, 24mM $NaHCO_3$, 4mM KCl, 1.8mM $CaCl_2$, 1mM $MgSO_4$, 0.43mM NaH_2PO_4 and 5.56mM glucose. As with cell culture the bronchioles are gassed with a 5% CO_2 to buffer against metabolic changes altering the pH of the solution thus maintaining a pH of 7.4. Presence of a responsive bronchiole was tested by adding 80mM KCl to the chamber. Thus inducing a contraction by depolarising the smooth muscle cell membrane and increasing the opening probability of voltage gated calcium channels, resulting in an influx of Ca^{2+} which activates the contractile apparatus via calmodulin. The positive control was repeated twice more and the final response was used to normalise subsequent agonist induced contractions as the bronchiole contraction settles and becomes more reproducible. The normalisation ensures it is the intrinsic

properties of the ASM within the bronchioles being compared and not merely the quantity present in each dissected section. The bronchioles were then washed back into Krebs solution, allowed to equilibrate for 30 minutes and then exposed to semi-log cumulative escalating concentrations of carbachol.

2.9 Statistics

All results are expressed as mean ± SEM. In most cases n numbers refer to the number of subjects, but in some studies the number of individual experiments. Duplicates/triplicates are defined as repeats from the same patient measured in parallel in the same experimental procedure to reduce inherent technical variations. Statistical analysis was carried out using SigmaPlot software and GraphPad Prism. When comparing means from more than two groups a one-way analysis of variance (one-way ANOVA) was used. Two or three-way analyses of variance were used when comparing the sample means of more than two groups and examining the influence of two/three categorical independent variables on one dependent variable. For example the influence of genotype and sex on airway hyper-responsiveness in knockout and wild type mice. Bonferroni's post-hoc test was used for the *in vitro* data and the Holm-Sidak post-hoc test for the *in* vivo data. Analysis of means and variance between just two groups was tested by Student's t-test. In all cases a p value of less than 0.05 was deemed as a significant difference between observed sample means and a statistical power of >0.8 was acceptable, further analysis is given where necessary by each result.

2.10 In vivo

All of the *in vivo* methods used in the *in vivo* investigation into reduced SERCA2 expression are outlined prior to the results in the corresponding chapter.

2.11 Materials

BDH Merck Ltd, Poole, U.K

Ethanol

Methanol

Tween-20

Becton, Dickison and Company

Yeast extract

Agar

Tryptone

Life Technologies™

(Fluo-4 NW kit)

Fungizone (amphotericin B)

Gentamicin

Non essential amino acids

Sodium pyruvate

L-glutamine

Dulbecco's modified Eagle's medium

Fetal bovine serum

Trypsin

Hank's balanced salt solution

MES running buffer

NuPAGE transfer buffer

Nitrocellulose membrane (0.2µm pore)

NuPAGE Bis-Tris Gel 1.0mm

NuPAGE sample buffer

Turbo DNase

pSilencer 3.0-H1

Promega

GloMax®-Multi Detection System

Millipore

Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel

Sigma-Aldrich Company Ltd, Poole, U.K

Bovine serum albumin

Insulin

Calponin clone DE-U-10

Desmin clone hCP

Smooth muscle α-actin clone 1ª4

Protease inhibitor

Urethane

Formoterol

Salmeterol

Forskolin

Phalloidin

Miscellaneous

^{3}H-thymidine (Amersham)

Ready-To-Go RT-PCR beads (GeHealthcare Life Technologies™, Buckingham, U.K)

DNeasy blood & tissue kit (QIAgen, West Sussex, U.K)

miRNeasy kit (QIAgen, West Sussex, U.K)

Kodak Biomax film MR1 (Perkin Elmer, Buckinghamshire, U.K)

BCA Protein assay kit (Pierce, Northumberland, U.K)

SERCA2 primary antibody (Abcam, Cambridge, U.K)

GAPDH primary antibody (Abcam, Cambridge, U.K)

Basic Nucleofector Kit Primary Smooth Muscle (LONZA, Basel, Switzerland)

Mulvany-Halpern type small vessel wire myograph (DMT, Denmark)

PCR master mix (Applied Biosystems)

Chapter 3 The effect of TNF-α, TGF-β and IL-13 on calcium handling proteins and homeostasis in ASM

3.1 Introduction

There is increasing evidence that calcium homeostasis is perturbed in the ASM in asthma (Mahn *et al.*, 2010). There are very few genetic risk factors associated with the disease that has the potential to alter this, with the exception of ORMDL3. Therefore the changes are believed to be a result of the inflammatory milieu altering the activity of the proteins controlling the homeostasis (Jia *et al.*, 2013; Mahn *et al.*, 2009; Sathish *et al.*, 2011; Sathish *et al.*, 2009; White *et al.*, 2006).

3.1.1 Inflammatory cytokines alter calcium handling proteins

Many of the altered phenotypes in asthma are dependent upon cytosolic calcium concentrations making it an important area to research and a potentially key site for therapeutic intervention. ASM cells derived from asthmatic patients display reduced protein expression of SERCA2 compared to healthy controls (Mahn *et al.*, 2009). The link between the activity of calcium handling proteins and the asthmatic phenotype are demonstrated clearly in this paper. A reduction in SERCA2 in healthy derived cells to levels to that seen in asthma using siRNA produces an asthmatic phenotype shown by enhanced proliferation, eotaxin-1 release and cell spreading (Mahn *et al.*, 2009).

Cytokines have the ability to mediate these changes in calcium handling. Overnight exposure to either TNF-α or IL-13 has been shown to decrease SERCA2 expression in human ASM cells (Sathish *et al.*, 2009). The change in protein expression was mirrored with a decline in function as a decrease in the rate of fall of $[Ca^{2+}]_i$ back to basal levels following agonist stimulation was observed in both treatment groups. This is believed to be an indicator of SERCA2 activity (Mahn *et al.*, 2009). In addition to altering SERCA2 expression treatment of ASM cells with TNF-α possibly results in a switch from receptor operated calcium entry (ROCE) to store operated calcium entry (SOCE) in TRPC3 dependent manner (White *et al.*, 2006). The asthmatic cytokines IL-13 and TGF-β act synergistically to increase the gene expression of TRPC6 and subtly increase TRPC3 (Karner, 2010). However no changes in their expressions were noted in cultured biopsies comparing healthy cells to asthmatic cells indicating the inflammatory milieu needs to be present.

The classical intracellular signalling cascade following TGF-β receptor activation involves the Smad family, a set of secondary messengers resulting in changes in gene transcription. Smad2 has been implicated in asthma as a murine model over expressing it in epithelial cells resulted in an enhanced asthmatic phenotype (Gregory *et al.*, 2010). Additionally a polymorphism in Smad3 leads to increased

prevalence of asthma, suggesting that it is involved in signalling pathways exacerbating the pathology (Moffatt *et al.*, 2010).

3.1.2 TRPA1

A recent paper showed that TRPA1 causes non-neurogenic airway inflammation and stained positive on ASM cells by immunohistochemistry (Nassini *et al.*, 2012). TRPA1 is a calcium permeable non selective cation channel like TRPC3 and TRPC6 and therefore has the potential to alter ASM phenotypes associated with asthma (Mahn *et al.*, 2010).

A collaboration was formed with Dr Andersson at King's College London whose expertise lay with the role of TRPA1 in nociception to make a preliminary assessment to the function of TRPA1 in ASM cells.

3.1.3 Aims

The aim of this chapter was to expand the current field of work by looking into the role of TNF-α, IL-13 and TGF-β in altering the protein expression of TRPC6, TRPC3 and SERCA2. Furthermore, to investigate whether the alterations in protein affect the functional handling of calcium in ASM cells and to elucidate possible signalling mediators controlling this.

In parallel to the main investigation in this chapter an assessment was carried out on cultured ASM cells to see whether TRPA1 ligands could induce calcium transients and secondly contraction on isolated murine bronchioles.

3.1.4 Hypotheses

I hypothesised that the exposure of the aforementioned inflammatory cytokines to healthy ASM cells will result in an up-regulation of TRPC6 and TRPC3 while diminishing SERCA2 expression. Ultimately leading to altered calcium signalling which underpins the asthmatic ASM phenotype.

Following the confirmation of TRPA1 on ASM cells (Nassini *et al.*, 2012), I hypothesised that their activation would result in a transient increase in intracellular calcium in cultured cells and a contraction of murine bronchioles.

3.2 Methods

Experiments were carried out as described in Chapter 2 on primary ASM lines derived from healthy patients.

The methylation data described in the discussion was performed by Dr Lavender's group following my preparation and stimulation of the cells.

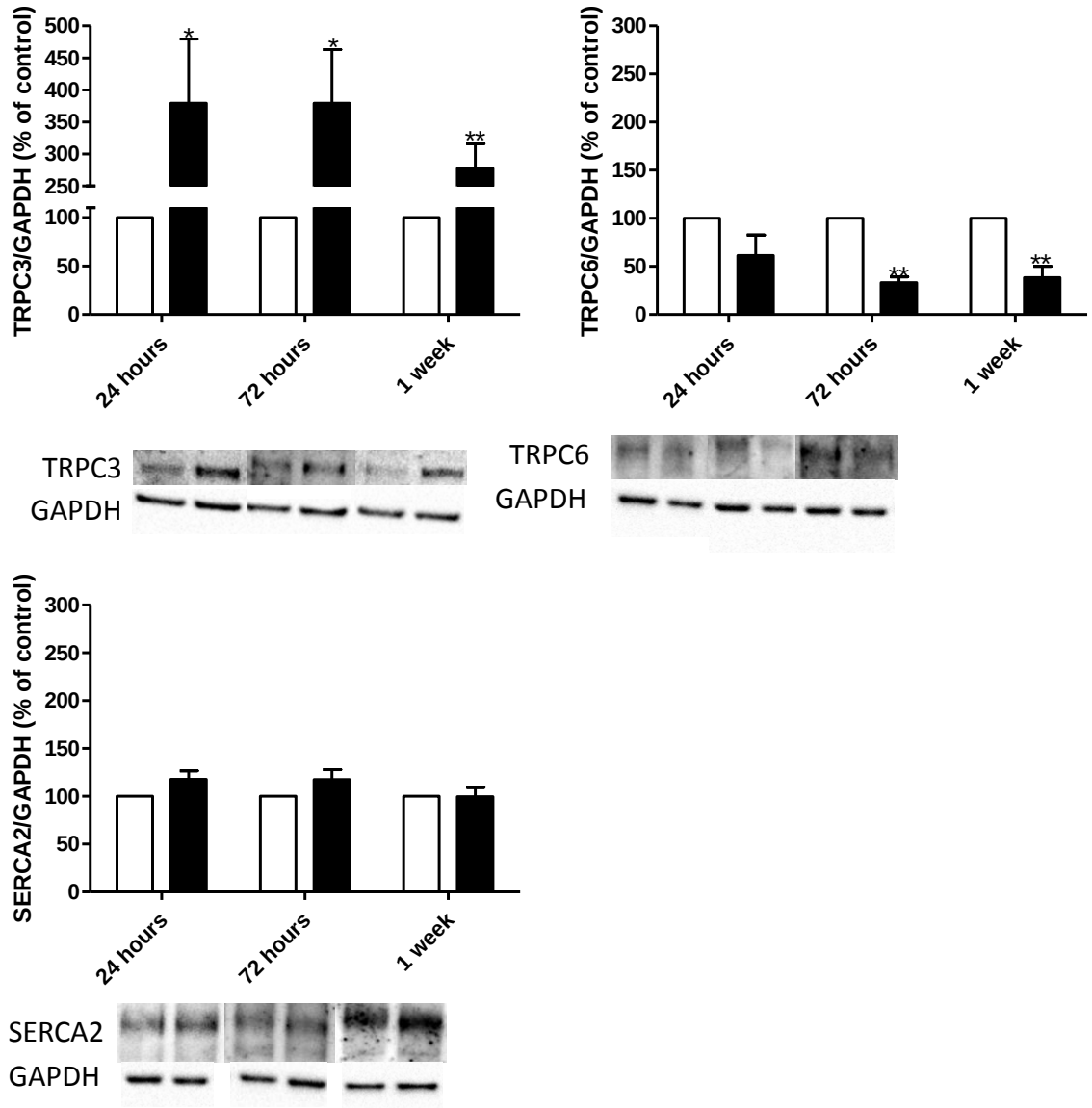
The TRPA1 calcium transients were performed by Dr Andersson following my preparation of the cells and the myography performed by Dr Prieto-Lloret with protocol consultation with Dr Andersson.

The range of time points were chosen because at 24 hours Ca^{2+} dependent responses in ASM cells have previously been shown to be altered by cytokine treatment (Deshpande *et al.*, 2004; Ojo, 2011). Up to one week time points were also investigated as asthma is a chronic disease and long term exposure of cytokines and growth factors have the potential to cause differential expression profiles compared to acute stimulation. The concentrations used have all been previously published within this field (Chen *et al.*, 2013; Mahn *et al.*, 2009; Matsumoto *et al.*, 2012; Sathish *et al.*, 2009).

3.3 The role of TNF-α in altering calcium homeostasis

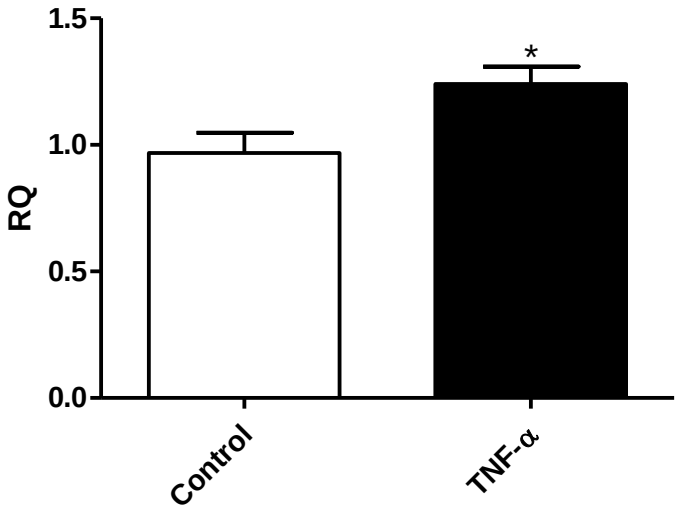
3.3.1 Changes in protein expression

Building upon the work carried out by Sathish *et al* (2009) the protein expression of TRPC3 and TRPC6 was also investigated following TNF-α stimulation (Fig. 3.3.1-1). TRPC3 protein expression was significantly elevated at all three time points with the most consistent elevation after a week (277 ± 38.8% of unstimulated control, $p=0.01$). Interestingly and conversely TRPC6 expression declined at every time point measured reaching significance at 72 hours and 1 week stimulation ($p<0.01$). SERCA2 expression was not significantly altered at any time point which lies in contrast to (Sathish *et al.*, 2009) where a decrease in expression was observed following overnight exposure.



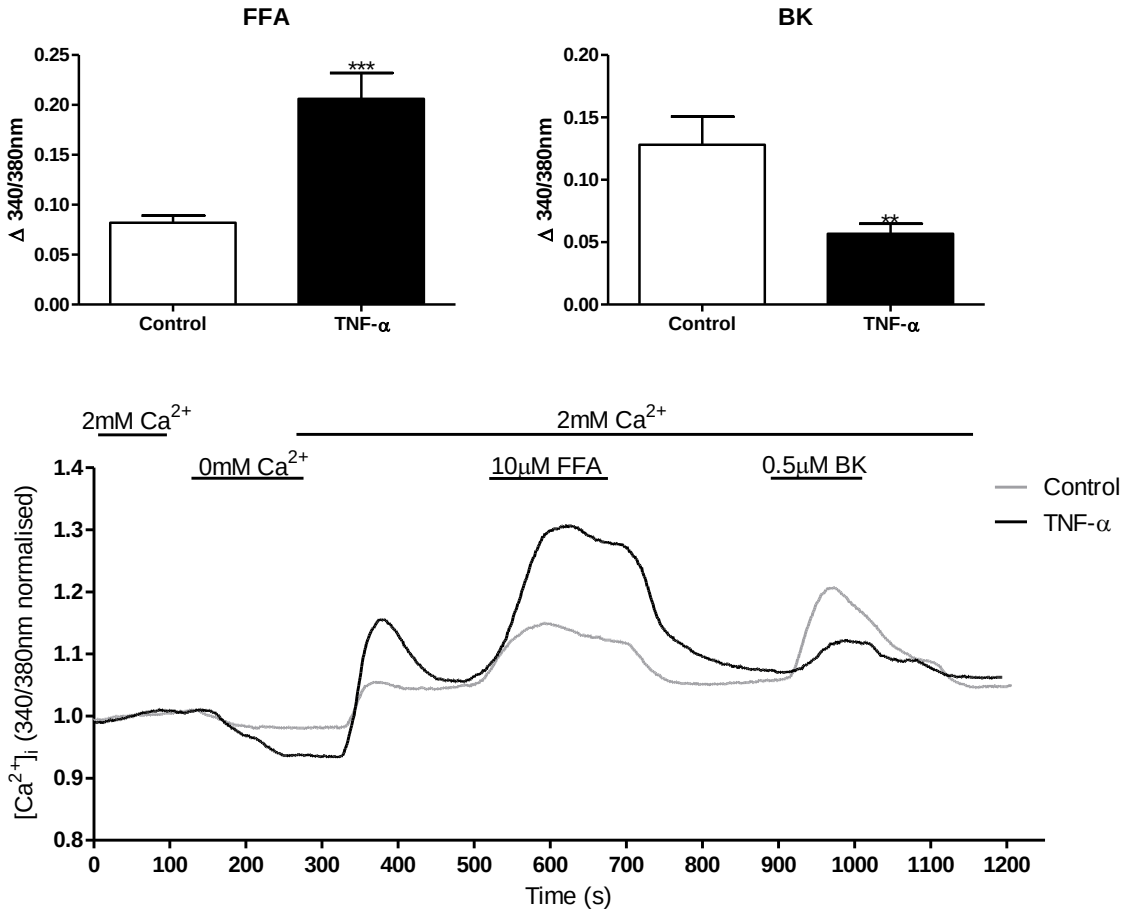
3.3.1-1 Healthy ASM cells were stimulated with 20ng.ml^{-1} TNF-α for either 24 hours, 72 hours or 1 week. A) Changes in TRPC6 expression, B) Changes in TRPC3 expression (all splices) and C) Changes in SERCA2 expression. Bars represent mean ± SEM, n=3-6, *p<0.05, **p<0.01 by unpaired t-test.

In order to clarify the conflicting results with (Sathish *et al.*, 2009) SERCA2 mRNA expression levels were measured following six hours stimulation with TNF-α. A small but significant increase in transcription was observed (Fig. 3.3.1-2, p=0.03) correlating with the very slight (but non-significant) rise in protein expression described in Fig. 3.3.1-1. ΔCt was obtained by subtracting 18S (control gene) average Ct values (cycle threshold) from the average Ct values from the gene of interest (SERCA2). RQ is the TGF-β treated ΔCT value normalised to its relevant control ΔCT value.



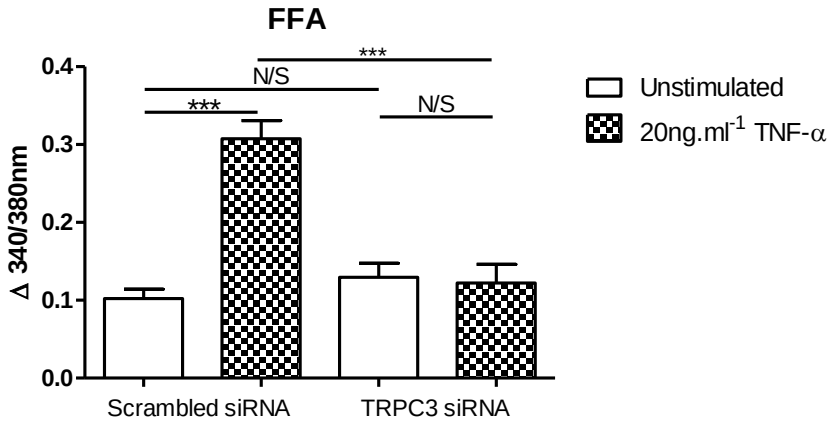
3.3.1-2 Changes in SERCA2 mRNA expression in response to stimulation for 6 hours with 20ng.ml^{-1} TNF-α in healthy ASM cells. Results were obtained by real time PCR and normalised to control, bars represent mean ± SEM (n=5, p=0.03) by unpaired t-test.

The changes in protein expression induced by 1 week of TNF-α on calcium handling proteins affects the functional handling of calcium within the cell. Cells treated with TNF-α have a significantly greater increase in intracellular calcium concentration upon stimulation with 10μM FFA, a putative TRPC6 agonist (Foster *et al.*, 2009) (Fig. 3.3.1-3, $p<0.001$). Upon stimulation with bradykinin, TNF-α treated cells had a significantly attenuated elevation in cytosolic calcium compared to untreated cells; 0.06 ± 0.001 Δ340/380 compared to control 0.13 ± 0.02 Δ340/380 ($p<0.01$).



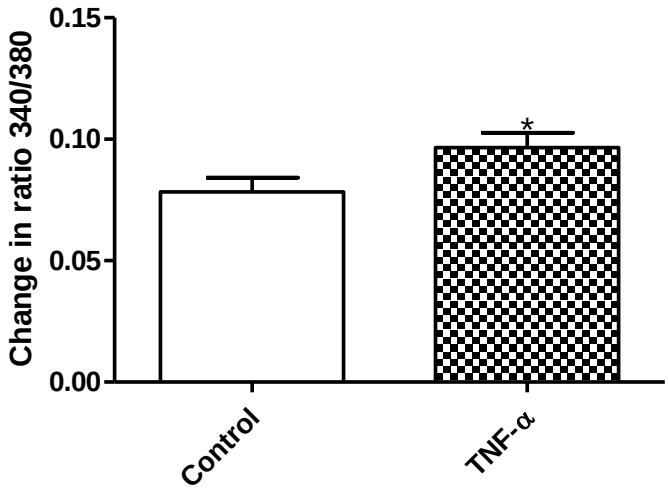
3.3.1-3 Ratiometric measurements of cytosolic calcium from control and 7 day 20ng.ml^{-1} TNF-α treated healthy ASM cells. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes and stimulating with **top left**; 10μM FFA or **top right**; 0.5μM BK, bars represent mean ± SEM, n=8-10, **p<0.01, ***p<0.001 by unpaired t-test. **Bottom**; example trace of protocol used.

The increase in cytosolic calcium elicited by FFA stimulation after TNF-α treatment is dependent upon TRPC3 protein expression. After reducing TRPC3 protein with siRNA no change occurs between the unstimulated control and TNF-α treated cells (Fig. 3.3.1-4).



3.3.1-4 Ratiometric measurements of cytosolic calcium from control (clear bars) or 7 day 20ng.ml^{-1} TNF-α (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or TRPC3 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes and stimulated with 10μM FFA. Bars represent mean ± SEM, n=9-10; ***p<0.001, by one-way ANOVA and Bonferroni's multi comparison post hoc test.

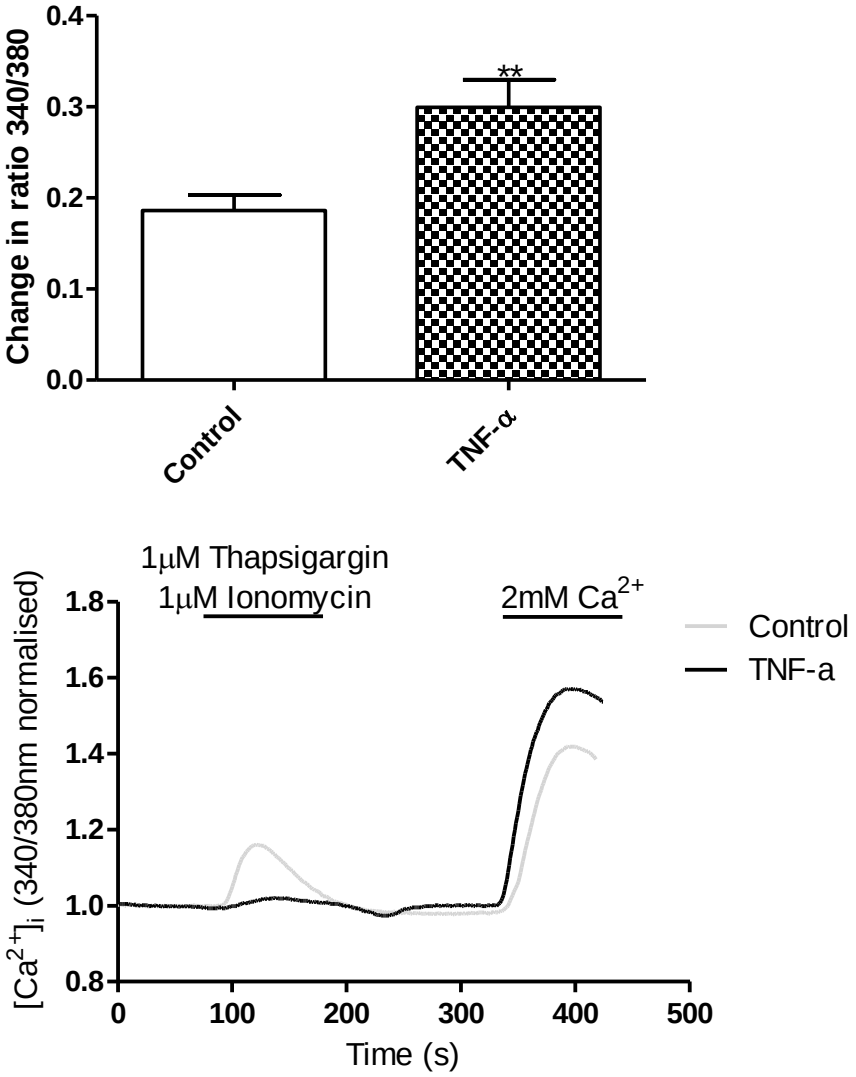
Upon removal of extracellular calcium from the perfusate an immediate fall in cytosolic calcium is observed suggesting a constitutively active calcium entry pathway (McVicker, 2003). It has previously been suggested that TRPC3 may play a role in this background non-selective calcium entry (Zhu *et al.*, 1998). Cells incubated for a week with TNF-α showed a small but significant increase in this basal entry pathway compared to the unstimulated control (Fig. 3.3.1-5, $p<0.05$). Combined with the increase in TRPC3 protein expression observed previously it is likely to be partially responsible for this current.



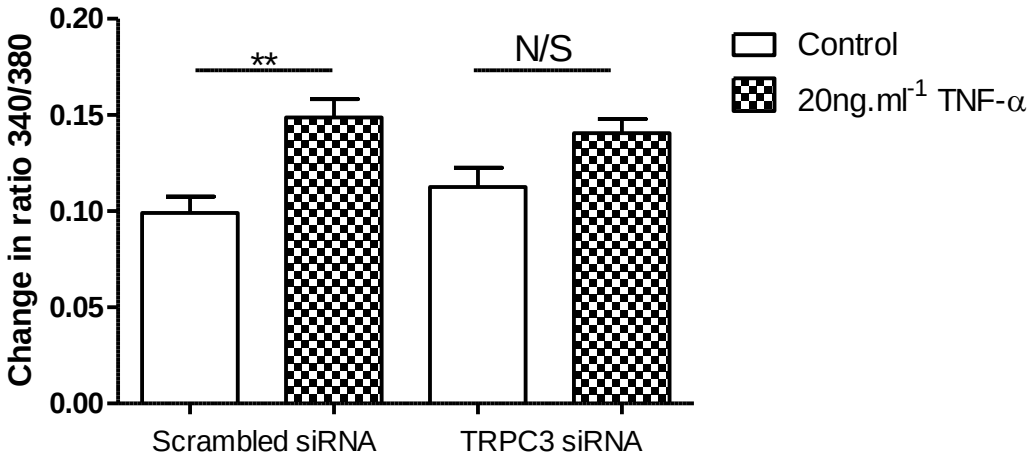
3.3.1-5 Ratiometric measurements of Fura PE 3-AM were used to determine the constitutive leak of calcium ions into the cytosol. Cells were treated with 20ng.ml^{-1} TNF-α for 7 days or control media. The change in ratio from a 2mM Ca^{2+} HBSS solution to a calcium free solution was measured, bars represent mean ± SEM, n=10-11, *p=0.05. Analysis performed by unpaired t-test.

3.3.2 Store operated calcium entry

Capacitative calcium entry (CCE) or store-operated calcium entry (SOCE) is triggered when the sarcoplasmic reticulum stores are emptied so that they can be replenished. An estimation of this can be made by removing extracellular calcium, depleting stores with ionomycin while preventing further reuptake with thapsigargin and then reintroducing calcium to the perfusate. Stimulating healthy ASM cells with TNF-α for one week increases SOCE compared to control; 0.30 ± 0.03 and 0.19 ± 0.02 Δ340/380nm respectively (Fig. 3.3.2-1, p=0.04). To test whether TRPC3 was responsible for the effect the experiment was repeated following transfection with a scrambled siRNA control and TRPC3 directed siRNA. The positive control was reproducible as TNF-α resulted in a significant increase in SOCE once more (p=0.003) (Fig. 3.3.2-2). The TRPC3 siRNA abolished the significant elevation caused by TNF-α (p=0.16) however it did not bring SOCE back to the control value. The power of the test was 0.937 with an α value of 0.05 therefore the result can be interpreted with confidence.



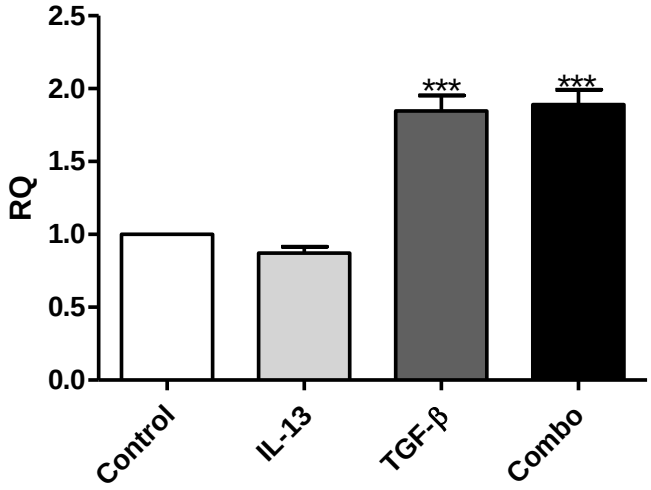
3.3.2-1 Ratiometric measurements of cytosolic calcium from control (clear bars) or 7 day 20ng.ml^{-1} TNF-α (chequered bars) treated healthy ASM cells. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes. Cells were then perfused with calcium free HBSS, intracellular calcium stores were emptied using 1 μM ionomycin and reuptake blocked with 1μM thapsigargin. SOCE was measured by the change in ratio of 340/380nm upon the reintroduction of calcium in the perfusion solution (above). Bars represent mean ± SEM, n=10-11, p=0.004 by unpaired t-test.



3.3.2-2 Ratiometric measurements of cytosolic calcium from control (clear bars) or 7 day 20ng.ml^{-1} TNF-α (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or TRPC3 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes. Cells were then perfused with calcium free HBSS, intracellular calcium stores were emptied using 1 μM ionomycin and reuptake blocked with 1μM thapsigargin. SOCE was measured by the change in ratio of 340/380nm upon the reintroduction of calcium in the perfusion solution Bars represent mean ± SEM, n=10-12; **p<0.01, by one-way ANOVA and Bonferroni's multi comparison post hoc test.

3.4 IL-13 and TGF-β

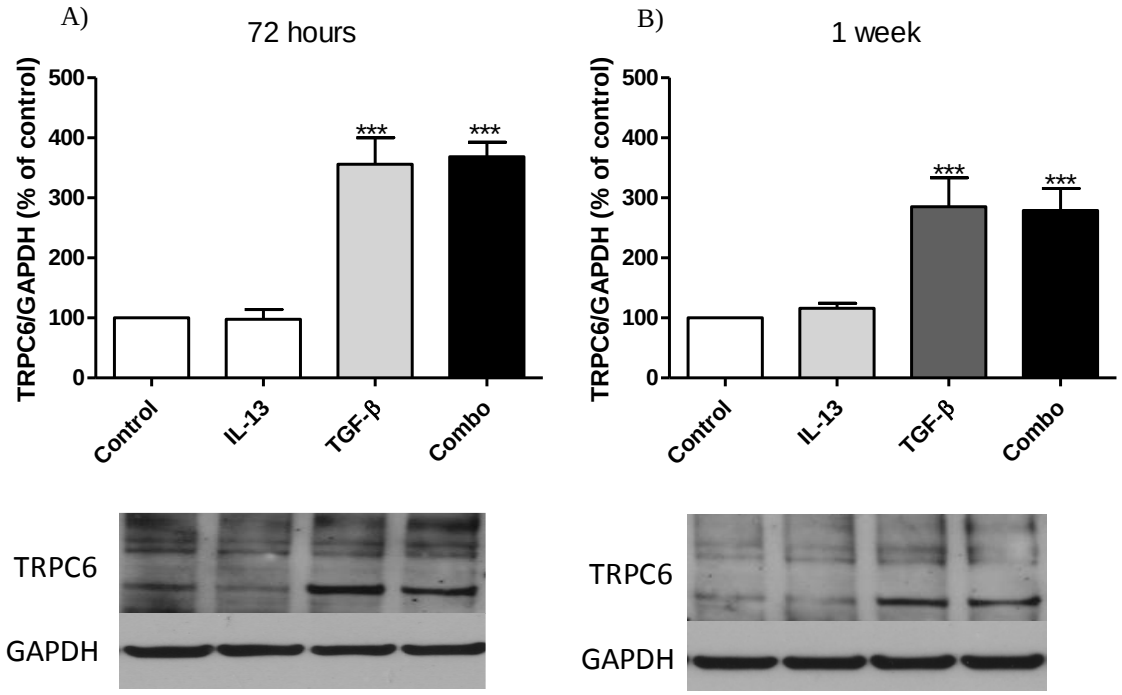
It has been published that treatment with a combination of IL-13 and TGF-β results in a synergistic increase in TRPC6 mRNA expression (Karner, 2010). In order to get a broader picture of the effect stimulation of these asthmatic cytokines have on the gene expression of calcium handling proteins SERCA mRNA was measured following stimulation. As with TRPC6 IL-13 stimulation alone had no effect whereas TGF-β caused a robust and significant increase. The combination of the two however had no additional effect compared to TGF-β alone (Fig. 3.3.2-1).



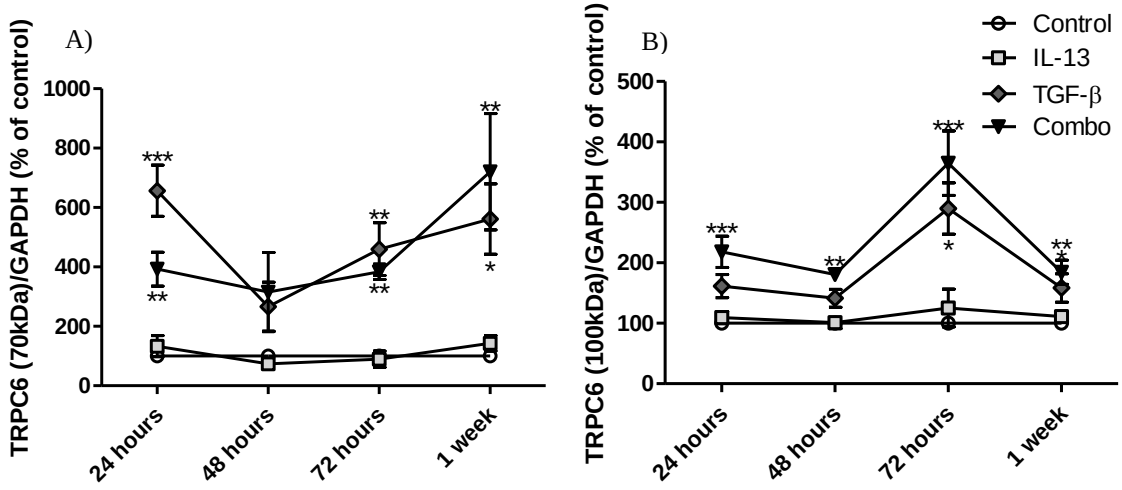
3.3.2-1 Changes in SERCA2 mRNA expression in response to stimulation for 6 hours with 10ng.ml^{-1} IL-13, TGF-β or a combination of both in healthy ASM cells. Results were obtained by real time PCR and normalised to control, bars represent mean ± SEM (n=6-7, $p<0.0001$ by one-way ANOVA and Bonferroni's multi comparison post hoc test).

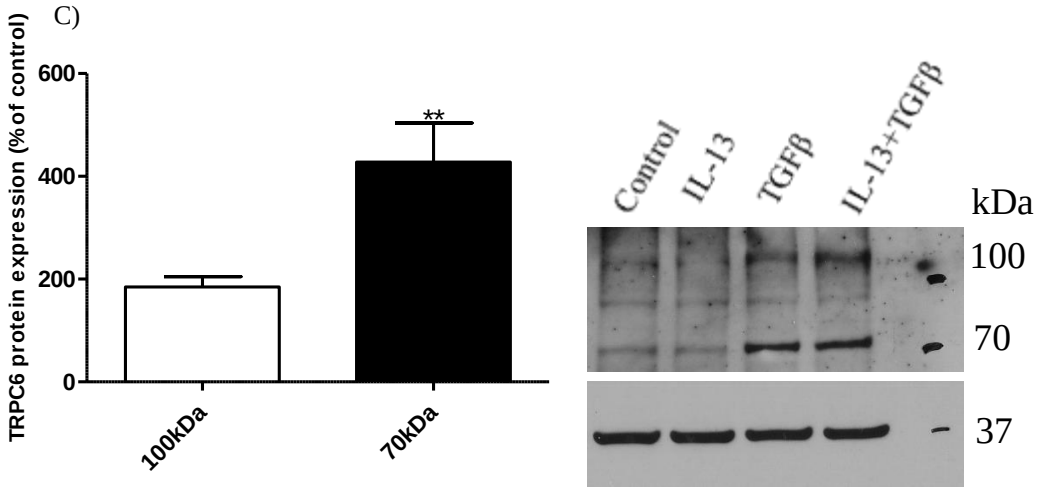
3.4.1 Protein expression

Treatment with 10ng.ml^{-1} TGF-β alone and in combination with 10ng.ml^{-1} IL-13 for both 72 hours and 7 days enhances TRPC6 protein expression (Fig. 3.4.1-1, $p<0.001$). In contrast to previous work from our lab (Karner, 2010) where a synergistic increase in mRNA was observed in the combined treatment group, there was no additional effect compared to TGF-β alone when measuring protein expression. Interestingly not all splices are affected evenly, after 7 days stimulation with the combination of IL-13 and TGF-β the 70kDa splice (accession number AK027769) increases more than the ≈100kDa splices (Fig. 3.4.1-2).



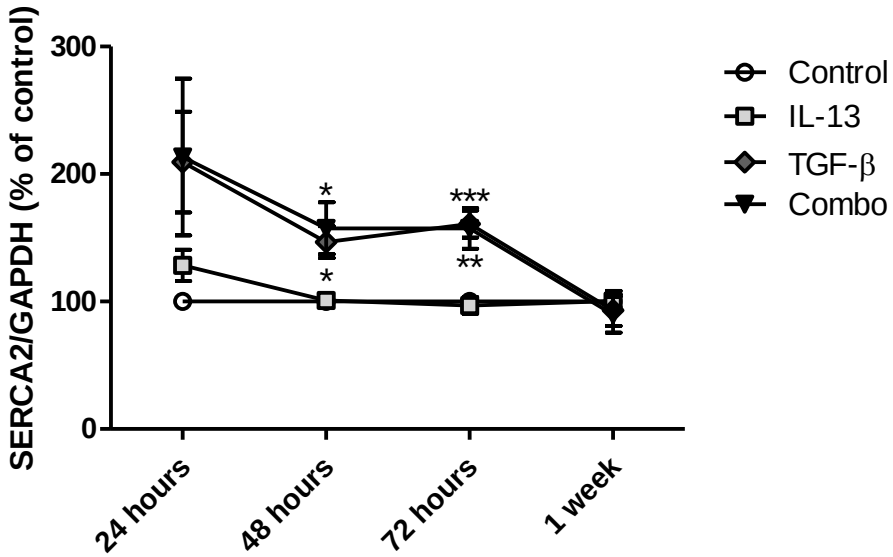
3.4.1-1 Healthy ASM cells were stimulated with 10ng.ml^{-1} IL-13, TGF-β or a combination of both for A) 72 hours or B) 1 week. Changes in total TRPC6 expression were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean, ± SEM, n=9-17 by one-way ANOVA and Bonferroni's multi comparison post hoc test.





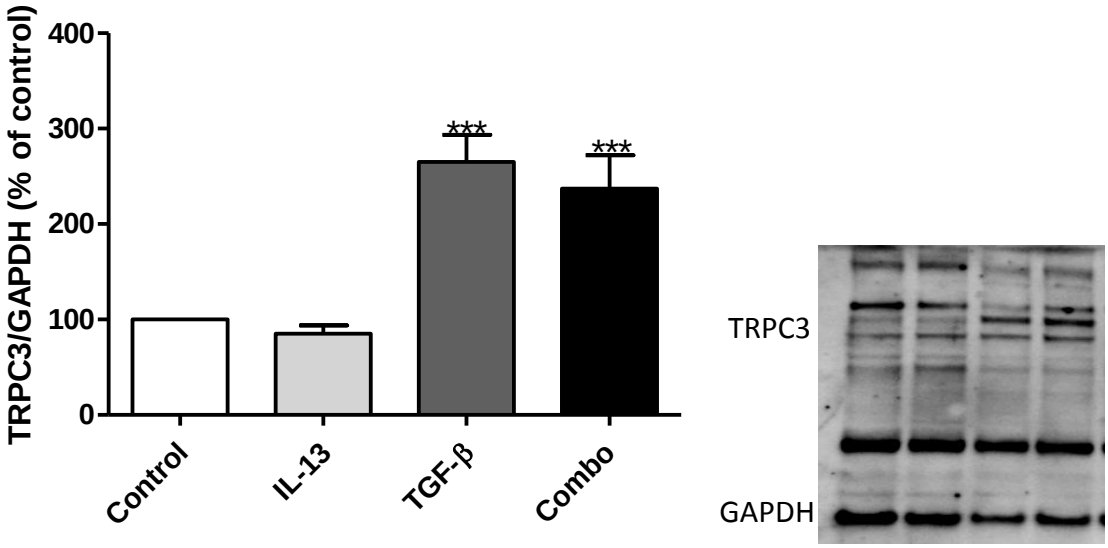
3.4.1-2 Healthy ASM cells were stimulated with 10ng.ml^{-1} IL-13, TGF-β or a combination of both for up to one week. Changes in the 70 and ≈100kDa TRPC6 splices' expression were measured by western immunoblot and expressed as a percentage of their unstimulated control, A and B respectively. Bars represent mean, ± SEM, n=2-9. C) A comparison between the up regulation of the 70kDa and ≈100kDa TRPC6 splices after one week combined IL-13 and TGF-β treatment with example trace, (n=5-9, p=0.01), by paired t-test.

Stimulation with TGF-β alone or in combination with IL-13 enhances SERCA2 expression up to 72 hours of stimulation after which it returns to control levels (48 hours $p<0.05$ for both, 72 hours $p<0.001$ combination, $p<0.01$ TGF-β, Fig. 3.4.1-3). IL-13 alone did not alter SERCA2 expression.



3.4.1-3 Healthy ASM cells were stimulated with 10ng.ml^{-1} IL-13, TGF-β or a combination of both for 24, 48, 72 hours or 1 week. Changes in SERCA2 expression were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean, ± SEM, n=5-14; *$p<0.05$, $p<0.01$ and ***$p<0.001$, by one-way ANOVA and Bonferroni's multi comparison post hoc test.

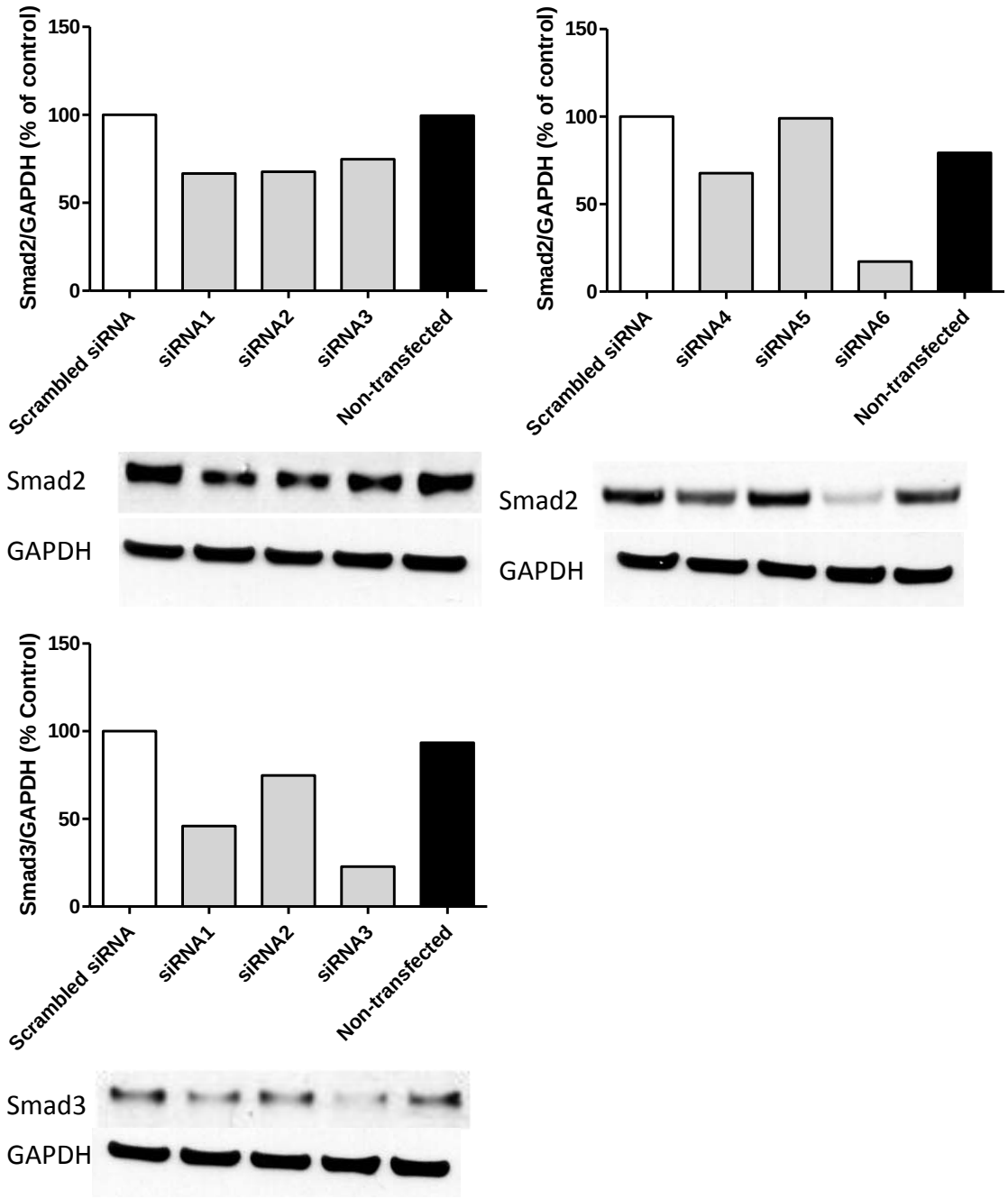
The effect of TGF-β on TRPC3 expression requires further investigation. While IL-13 did not affect TRPC3 expression it appeared as though TGF-β significantly increased expression ($p<0.001$). However as can be observed from the example blot there is a band the TRPC3 antibody detects above the suspected TRPC3 band showing the exact opposite effect to TGF-β. The band increasing in expression was chosen for analysis as it matches the real-time PCR data. Work is currently being carried out by Dr Shaifta to ascertain whether the correct band has been analysed by over-expressing TRPC3 tagged to green fluorescence protein (GFP) and by knocking TRPC3 mRNA expression down with siRNA.



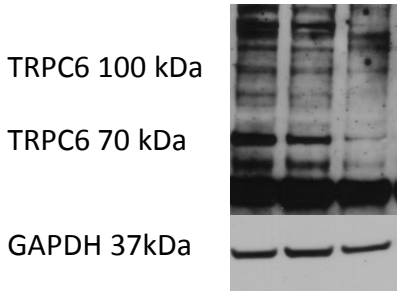
3.4.1-4 Healthy ASM cells were stimulated with 10ng.ml^{-1} IL-13, TGF-β or a combination of both for 1 week. Changes in TRPC3 expression were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean, ± SEM, n=10; ***$p<0.001$, by one-way ANOVA and Bonferroni's multi comparison post hoc test.

3.4.2 The role of Smad2/3 in the effects mediated by TGF-β

In order to investigate whether the effects of TGF-β on calcium handling proteins is mediated via Smad2 and/or Smad3, siRNAs targeting them were designed as described in section 2.6.1. Three different coding sequences were designed against each mRNA sequence initially and healthy ASM cells were transfected with them via electroporation using the Amaxa Nucleofector® kit. Protein levels were then analysed by western blot to determine which siRNA reduced protein expression most efficiently. A scrambled siRNA sequence was transfected and measured alongside the tested siRNAs as well as a non-transfected control to ensure the electroporation did not affect protein expression. Fig. 3.4.2-1 shows that for Smad2 none of the initial three sequences tested gave a sufficient knockdown of the protein so another three were designed and tested. Of the second set siRNA6 gave an 83% reduction compared to control. For Smad3 siRNA3 gave the best knockdown of 77%, for all future experiments these two siRNAs were used.



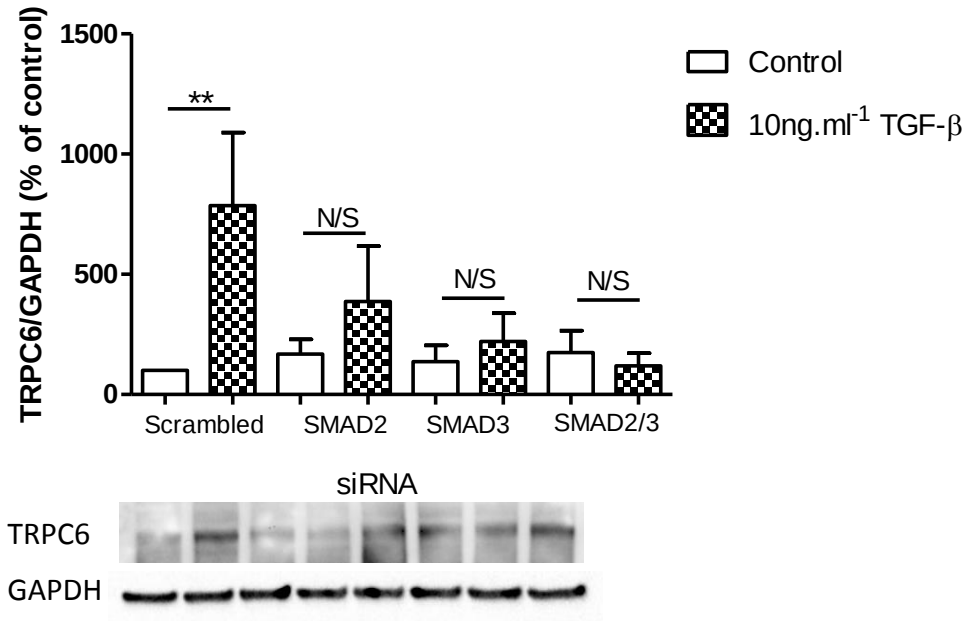
3.4.2-1 Healthy ASM cells were transfected via electroporation with 3µg of three different siRNA sequences targeted against A) Smad2 or B) Smad3, along with a scrambled siRNA sequence and just the nucleofector solution alone as controls. Western blots were then carried out on the lysed cells to measure the efficiency of each siRNA construct. Bars represent protein levels as a percentage of the scrambled siRNA control, n=1.



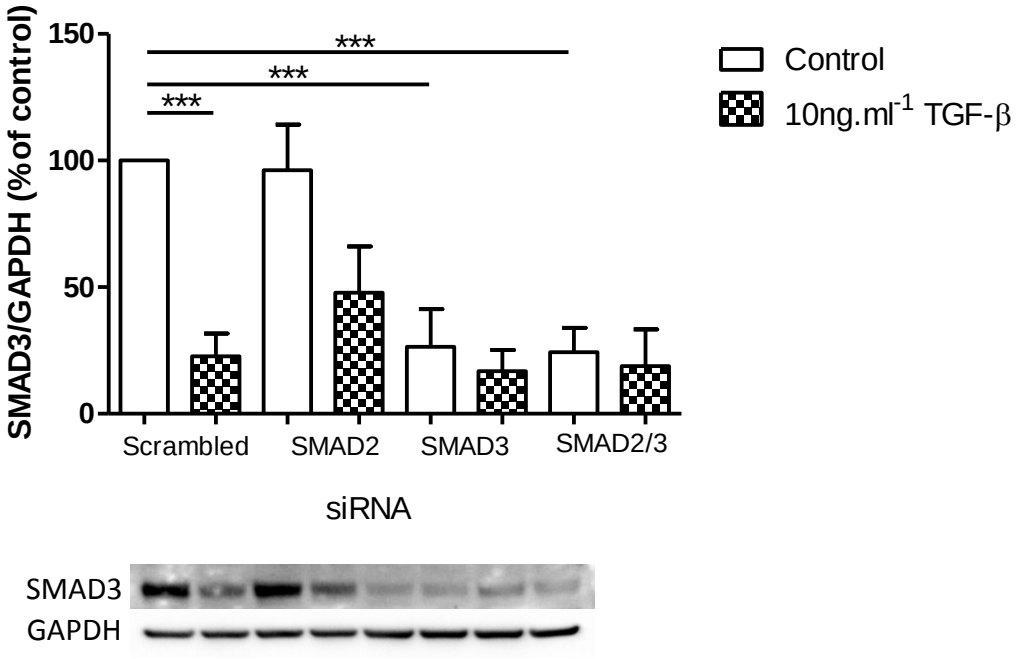
3.4.2-2 An example blot demonstrating the TRPC6 siRNA designed by Dr Shaifta knocks down both the 100kDa and 70kDa splices in healthy ASM cells. Lane 1 - untransfected control, lane 2 - scrambled siRNA and lane 3 - TRPC6 targeted siRNA.

3.4.3 The role of Smad2/3 in TGF-β signalling in ASM cells

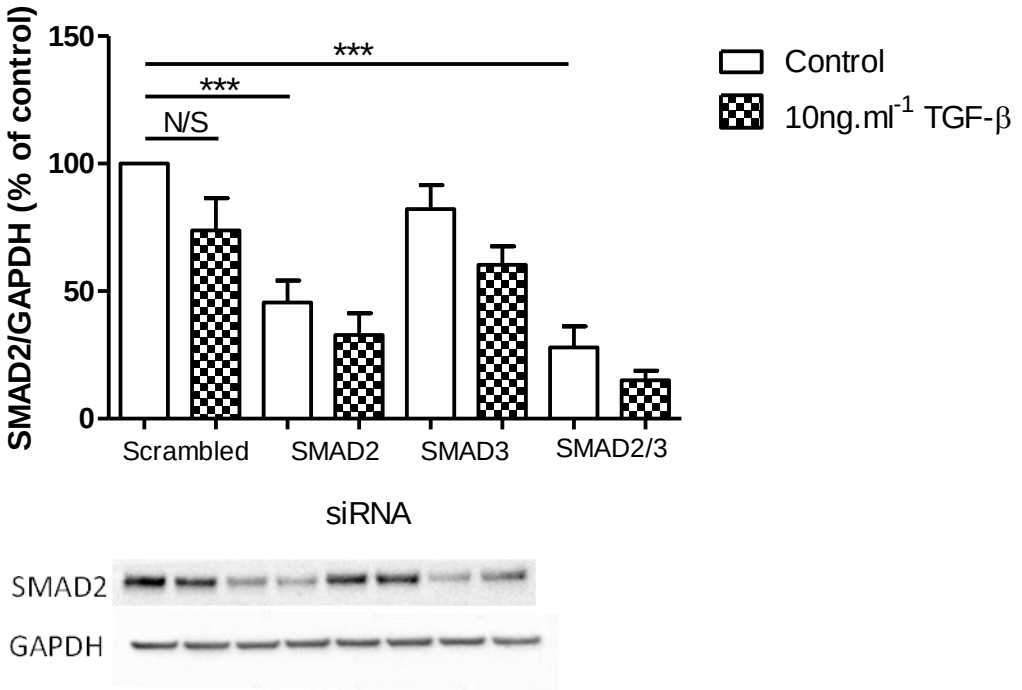
As before, TGF-β stimulation for 72 hours increases TRPC6 protein expression, (7.9 fold increase compared to control, $p<0.01$). The effect is partially attenuated by the Smad2 siRNA (2.3 fold increase compared to control, not significant) and further diminished by the Smad3 siRNA (1.6 fold increase, not significant). When the two are combined together TRPC6 protein expression doesn't increase at all following TGF-β stimulation (Fig. 3.4.3-1). To ensure the transfection had worked efficiently Smad3 and Smad2 expression levels were checked (Figs. 3.4.3-2 and 3.4.3-3 respectively). Both proteins were significantly reduced when targeted with siRNA ($p<0.001$) and interestingly Smad3 was also reduced by TGF-β stimulation alone ($p<0.001$), perhaps as part of a negative feedback loop to prevent excessive signalling. A full length time course was carried out to confirm this effect (Fig. 3.4.3-4). Little cross-reactivity between the siRNAs was observed indicating their specificity.



3.4.3-1 Healthy ASM cells were transfected with either scrambled, Smad2 targeted, Smad3 targeted or a combination of Smad2/3 targeted siRNA and cultured in the presence or absence of $10ng.ml^{-1}$ TGF-β for 72 hours. Total TRPC6 protein expression was then measured. Data represents mean ± SEM, n=4, **$p<0.01$ by one-way ANOVA and Bonferroni's multi comparison post hoc test.

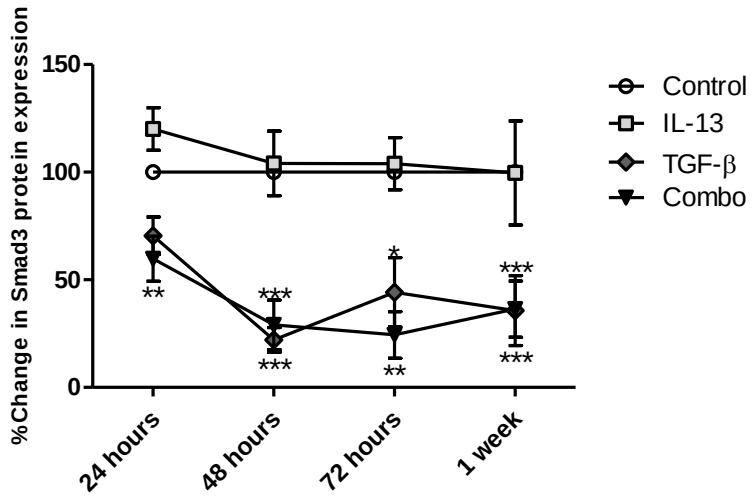


3.4.3-2 Healthy ASM cells were transfected with either scrambled, Smad2 targeted, Smad3 targeted or a combination of Smad2/3 targeted siRNA and cultured in the presence or absence of 10ng.ml^{-1} TGF-β for 72 hours. Total Smad3 protein expression was then measured. Data represents mean ± SEM, n=3, ***p<0.001 by one-way ANOVA and Bonferroni's multi comparison post hoc test.



3.4.3-3 Healthy ASM cells were transfected with either scrambled, Smad2 targeted, Smad3 targeted or a combination of Smad2/3 targeted siRNA and cultured in the presence or absence of 10ng.ml^{-1} TGF-β for 72 hours. Total Smad2 protein expression was then measured. Data represents mean ± SEM, n=5, ***p<0.001 by one-way ANOVA and Bonferroni's multi comparison post hoc test.

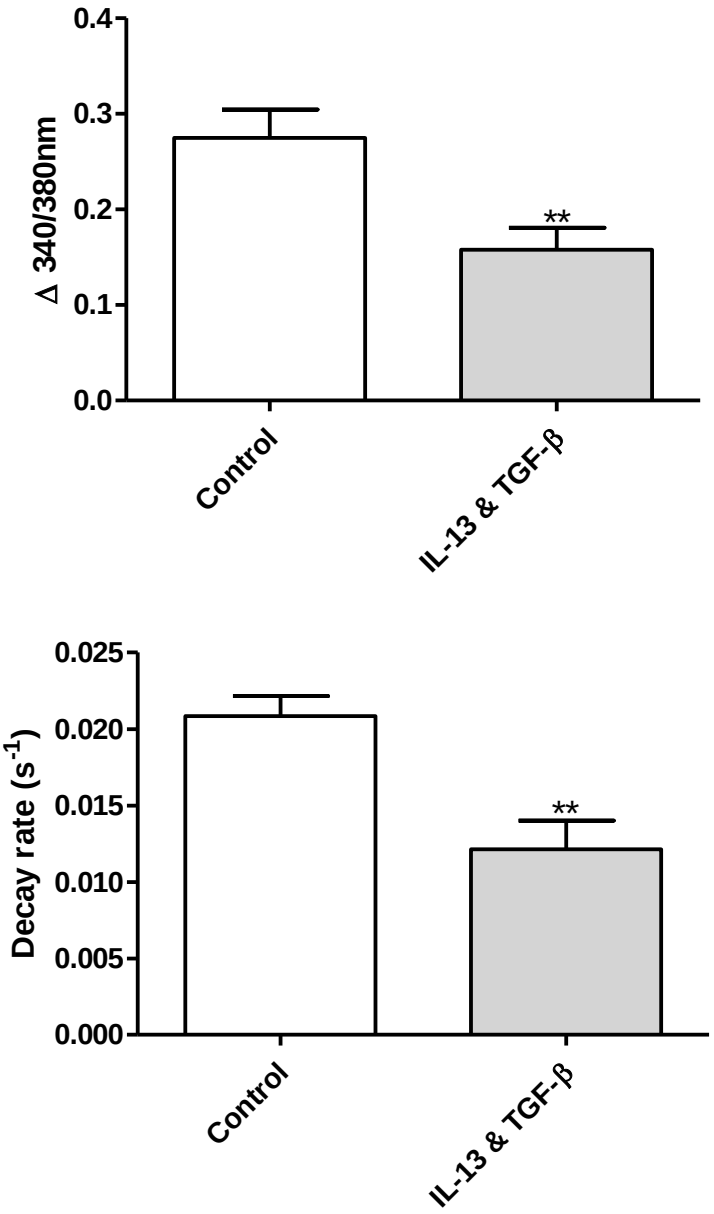
Treatment with TGF-β alone significantly decreased Smad3 expression in ASM cells as measured by western blot at 48 hours, 72 hours and 7 days ($p<0.001$, $p<0.05$ and $p<0.001$ respectively). IL-13 had no effect on Smad3 expression alone and when combined with TGF-β the effect was comparable to that of TGF-β alone (Fig. 3.4.3-4).



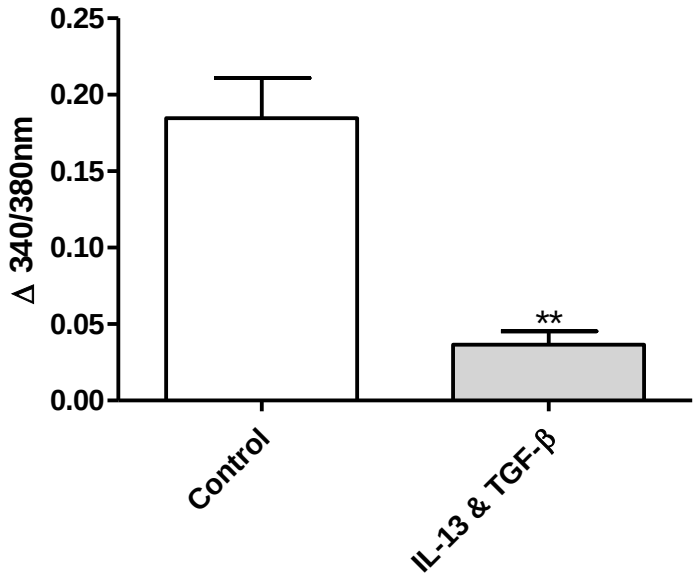
3.4.3-4 Healthy ASM cells were stimulated with 10ng.ml^{-1} IL-13, TGF-β or a combination of both for 24, 48, 72 hours or 1 week. Changes in Smad3 expression were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean, ± SEM, n=4-7; *$p<0.05$, $p<0.01$ and ***$p<0.001$ by one-way ANOVA and Bonferroni's multi comparison post hoc test.

3.4.4 Functional consequences

Following on from the observed changes in protein expression in various calcium handling proteins after stimulation with IL-13 and TGF-β it was important to find out whether this translated into a functional effect. Pre-treatment with a combination of IL-13 and TGF-β for one week significantly reduced the response to both bradykinin ($p=0.0068$, n=9, Fig. 3.4.4-1 upper) and FFA ($p=0.002$, n=4-5, Fig. 3.4.4-2) contrasting the TNF-α stimulation. The recovery rate back to the baseline value following BK stimulation was significantly slower following one week TGF-β and IL-13 treatment compared to control ($p=0.002$, n=7-8, Fig. 3.4.4-1 lower).

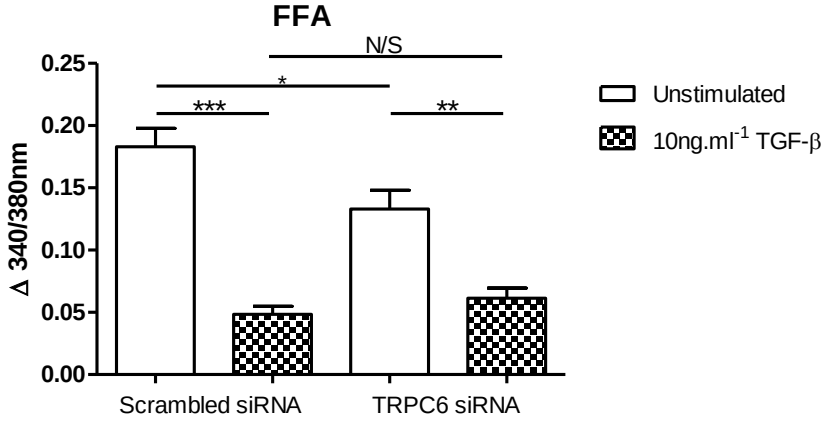


3.4.4-1 Ratiometric measurements of cytosolic calcium from control (clear bar) or 7 day IL-13 and TGF-β stimulation (grey bar). Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes. Cells were stimulated with 0.5μM bradykinin, ratiometric measurements (340/380nm) of peak calcium store release (top) and decay rate of signal (bottom). Bars represent mean and SEM, (n=7-9, **p<0.01) by unpaired t-test.



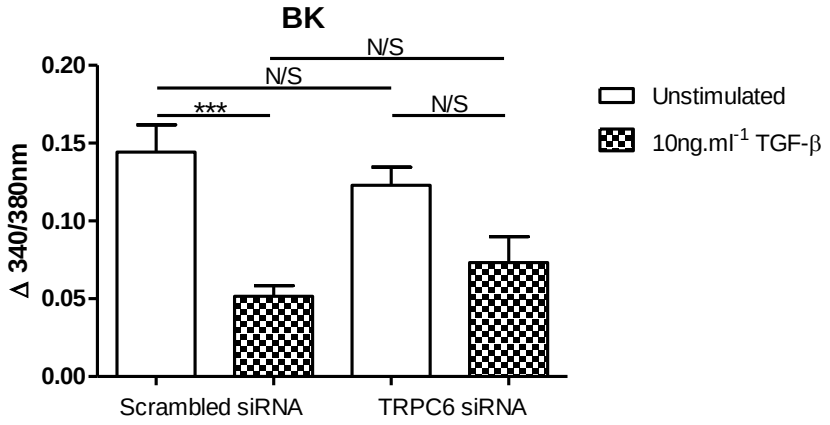
3.4.4-2 Ratiometric measurements of cytosolic calcium from control (clear bar) or 7 day IL-13 and TGF-β stimulation (grey bar). Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes. Ratiometric measurements of peak calcium store release following activation by 10μM FFA. Bars represent mean and SEM, (n=4-5, p=0.002) by unpaired t-test.

Transfection with a TRPC6 directed siRNA caused a small but significant reduction in FFA mediated rise in calcium ($p<0.05$) suggesting the effect was in part via TRPC6. The TRPC6 directed siRNA combined with TGF-β treatment caused no further reduction in FFA mediated calcium mobilisation compared to TGF-β, scrambled transfected cells (Fig. 3.4.4-3).



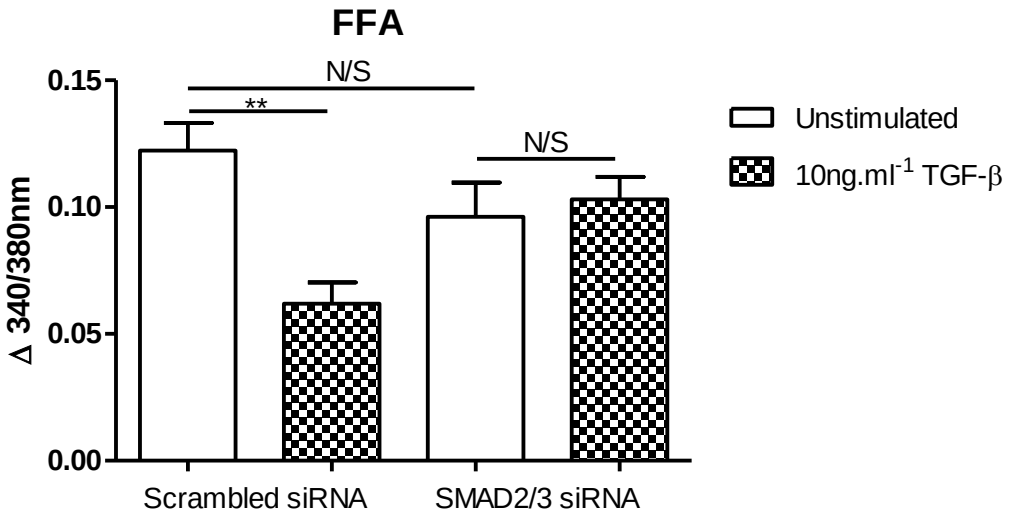
3.4.4-3 Ratiometric measurements of cytosolic calcium from control (clear bars) or 10ng.ml^{-1} TGF-β (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or TRPC6 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes and stimulated with 10μM FFA. Bars represent mean ± SEM, n=6-10; *p<0.05, p<0.01 and ***p<0.001 by one-way ANOVA and Bonferroni's multi comparison post hoc test.

Consistent with the previous run TGF-β treatment reduced calcium mobilisation in response to BK ($p<0.001$). With the addition of TRPC6 siRNA the control response was blunted and furthermore lessened the reduction induced by TGF-β. Both effects were to such an extent that the difference between them was lost, suggesting a potential role for TRPC6 in the BK response.

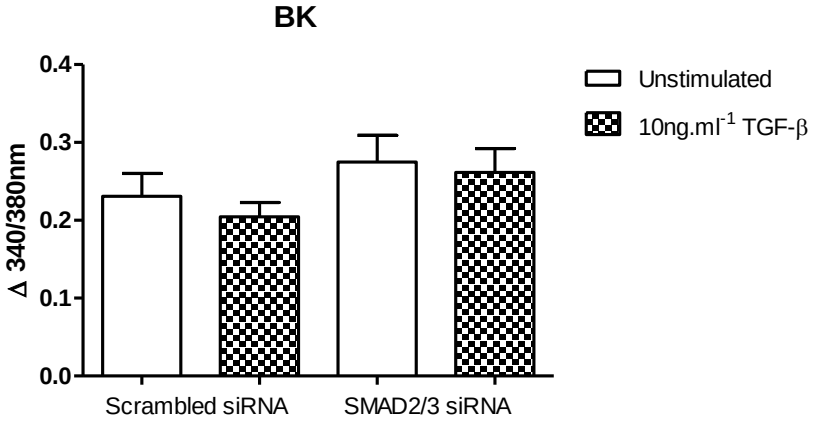


3.4.4-4 Ratiometric measurements of cytosolic calcium from control (clear bars) or 10ng.ml^{-1} TGF-β (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or TRPC6 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1μM Fura PE 3-AM for >45minutes and stimulated with 0.5μM BK. Bars represent mean ± SEM, n=6-10; *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA and Bonferroni's multi comparison post hoc test.

Combination of the Smad2/3 siRNAs significantly inhibits the increase of TRPC6 protein expression following TGF-β stimulation to control levels. To determine whether the reduction in FFA and bradykinin response was a Smad2/3 mediated effect, cells were transfected with the combination of siRNAs prior to TGF-β stimulation. The reduction in response following stimulation was completely abolished thus highlighting the importance of Smad2/3 in the observed effect (Fig. 3.4.4-5). When exploring the role of Smad2/3 following bradykinin stimulation, the positive control previously showing a reduced response did not work therefore no insights could be made (Fig. 3.4.4-6).



3.4.4-5 Ratiometric measurements of cytosolic calcium from control (clear bars) or 10ng.ml^{-1} TGF-β (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or a combination of Smad2/3 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1µM Fura PE 3-AM for >45minutes and stimulated with 10µM FFA. Bars represent mean ± SEM, n=9-10; **p<0.01 by one-way ANOVA and Bonferroni's multi comparison post hoc test.

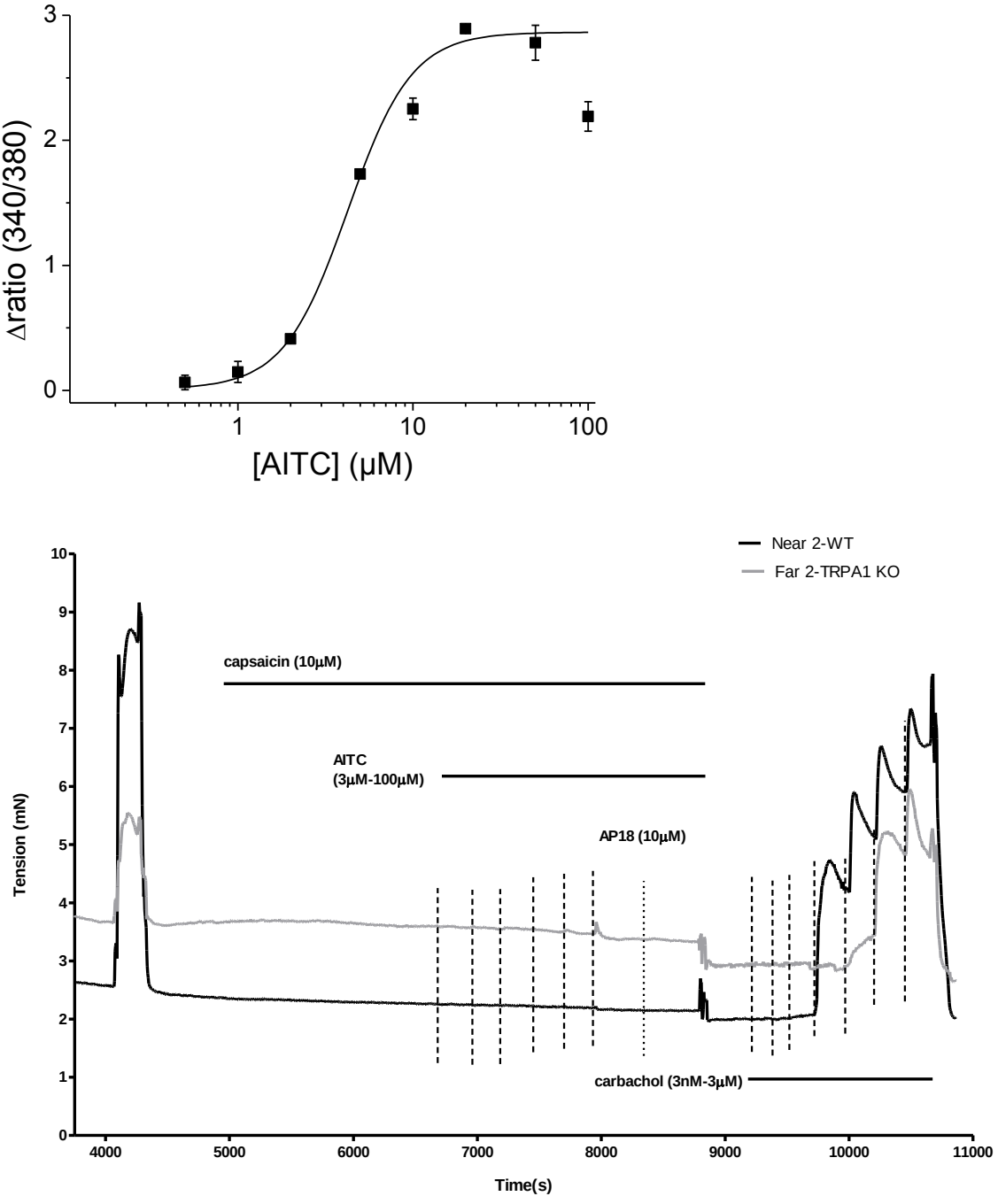


3.4.4-6 Ratiometric measurements of cytosolic calcium from control (clear bars) or 10ng.ml^{-1} TGF-β (chequered bars) treated healthy ASM cells. Prior to stimulation cells were either transfected with scrambled siRNA or Smad2/3 targeted siRNA. Cytosolic calcium ion levels were measured by preloading cells with 1µM Fura PE 3-AM for >45minutes and stimulated with 0.5µM BK. Bars represent mean ± SEM, n=9-10; *p<0.05, p<0.01 and ***p<0.001 by one-way ANOVA and Bonferroni's multi comparison post hoc test.

3.4.5 A role for TRPA1 in ASM

A dose response was performed on cultured healthy ASM cells with the TRPA1 agonist allyl isothiocyanate (AITC) measuring the peak rise in intracellular calcium elicited giving an EC_{50} of 3.5µM (Fig. 3.4.5-1 top). Secondly a dose response curve of AITC was performed on murine primary bronchioles mounted onto a myograph to investigate whether the rise in intracellular calcium observed in human cultured ASM would translate into a contraction. Both wild type and TRPA1$^{-/-}$ mice were used to determine the specificity of the

response. Following pre-incubation with the TRPV1 agonist capsaicin to desensitise the receptor a dose response to AITC was performed. No contractile response was observed in any of the mice for either the wild types or knockouts (Fig. 3.4.5-1 bottom).



3.4.5-1 Top: Healthy human ASM cells were grown in culture to sub confluence on 96 well plates, serum starved for 72 hours and loaded with fura-2 dye for 45 minutes. A dose response to allyl isothiocyanate (AITC) a TRPA1 agonist was performed. Dose response curve is from 12 replicates from the same cell donor. Bottom: Murine primary bronchioles from three wild type and three TRPA1$^{-/-}$ mice were stimulated with 10μM capsaicin for 30 minutes to desensitise TRPV1 channels followed by a dose response to AITC. A positive control of 80mM K^+ was performed prior to the experiment and a dose response to carbachol at the end. One representative trace is shown from each genotype of mouse.

3.5 Discussion

The results in this chapter show that the inflammatory cytokines TGF-β and TNF-α alter the expression of calcium handling proteins in healthy ASM and that the observed changes have a functional effect. I hypothesised that all three of TNF-α, TGF-β and IL-13 would increase the expression of TRPC3 and TRPC6 while diminishing SERCA2 expression resulting in an elevated $[Ca^{2+}]_i$ at rest and following agonist stimulation. Part of this hypothesis was proven correct as TGF-β enhanced TRPC6 and TRPC3 protein expression and TRPC3 was also increased by TNF-α. Contrary to the hypothesis IL-13 had no synergistic effect when co-stimulated with TGF-β and SERCA2 protein expression was not decreased by any of the treatments. The functional result of this was an increase in calcium leak, SOCE and FFA induced calcium entry and a reduction in bradykinin stimulated calcium mobilisation to TNF-α. As well as a Smad2/3 dependent decrease in FFA induced calcium entry and a reduction in bradykinin stimulated calcium mobilisation to TGF-β.

3.5.1 Effects of TNF-α on Ca^{2+} handling proteins and homeostasis

Exposure to TNF-α results in an up-regulation of TRPC3 protein expression and an increase in the peak calcium response to FFA and the basal leak of Ca^{2+} into the cell. Prior transfection with a TRPC3 directed siRNA abolishes the increase to FFA entirely therefore suggesting the influx of calcium is linked to this channel. TNF-α stimulation also resulted in a decrease in bradykinin elicited cytosolic calcium mobilisation. These results are in partial accordance with (White *et al.*, 2006), who also showed TNF-α enhancing TRPC3 mRNA and protein expression. The paper further demonstrated a higher resting cytosolic Ca^{2+} concentration following TNF-α stimulation which was dependent upon the presence of extracellular Ca^{2+} and TRPC3. Here it is shown that incubation with TNF-α increases the basal leak of Ca^{2+} from the extracellular perfusate into the cell. The combination of the results strongly indicates that TRPC3 is constitutively active allowing a basal flow of calcium ions into the cell, thus opening up the possibility of an inverse agonist being used for therapy should TRPC3 be highlighted as a candidate target. As highlighted in the introduction TRPC3 is only monoglycosylated in the extracellular loop leading to higher constitutive activity compared to the dually glycosylated TRPC6 (Dietrich *et al.*, 2003). In contrast to the White *et al.*, 2006 paper TNF-α resulted in a decreased agonist induced calcium mobilisation was observed. The augmented response published has been previously shown (Amrani *et al.*, 1995) but again at an earlier 24 hour stimulation time point compared to the 7 day one used in the present study. The same lab showed that 24 hours of TNF-α stimulation leads to decreased SERCA2 expression (Sathish *et al.*, 2009) which

would be expected to decrease SR store content and therefore result in a smaller rise in calcium following agonist stimulation as observed presently. A decrease in SERCA2 activity at 24 hours or the 7 day time point tested here was not observed however a decrease in TRPC6 was which has been attributed to receptor operated calcium entry and may explain the discrepancy in the results.

3.5.2 Store operated calcium entry (SOCE)

SOCE is the process of re-filling calcium stores following agonist stimulation in ASM cells and has been postulated to play a role in bronchial remodelling (Sweeney *et al.*, 2002).

SOCE following the emptying of the sarcoplasmic reticulum and adding calcium back to the extracellular solution was enhanced with TNF-α stimulation. Numerous proteins have been implicated with this phenomenon including stromal interaction molecule 1 (STIM1) and Orai1 (Peel *et al.*, 2008; Zhang *et al.*, 2005). Recent data has shown the inflammatory cytokines TNF-α and IL-13 induce a constitutive increase in SOCE in ASM cells by increasing STIM1 puncta size (Jia *et al.*, 2013). This paper postulates STIM1 interacts with the plasma membrane protein Orai1 to facilitate SOCE as depletion of Orai1 via siRNA blunts the response. TNF-α also increases caveolin-1 and Orai1 expression while forming more caveolae, thus enhancing the interaction between the plasma membrane (Orai1) and SR (STIM1) and facilitating SOCE (Sathish *et al.*, 2012). To elucidate the role of TRPC3 the cells were transfected with siRNA prior to stimulation and the elevation in SOCE following TNF-α stimulation was no longer observed as has been shown previously for a much shorter stimulation (White *et al.*, 2006). The response was still subtly enhanced following TNF-α stimulation and TRPC3 knock down, suggesting that TRPC3 might not a necessary component of SOCE. Testing with the TRPC6 siRNA would be interesting as it has been shown to interact with Orai1 (Liao *et al.*, 2007) to evaluate the role of the TRPC family in SOCE as none may be a necessity but rather used as a method of control during inflammation. Their presence and the ability of STIM1 puncta size to increase following TNF-α stimulation may be just a regulatory mechanism.

Distinguishing the roles of TRPC3 and TRPC6 between each other as well as in receptor or store operated calcium entry in ASM cells has historically been a difficult task to undertake. A poor availability of specific pharmacological tools and antibodies has stifled advances so far. Gene expression analysis has shown that TRPC6 is expressed at approximately 30 fold higher levels than TRPC3 in healthy cultured ASM (Karner, 2010). TNF-α stimulation affected TRPC6 conversely to TRPC3 causing a robust decrease in expression lying in contrast to the findings observed by White *et al.*, 2006 where they observed no change. The discrepancy is likely due to the differing time points observed as they looked at 18-22 hours post stimulation and at 24 hours we saw an increase which was quite variable. The longer time point of 1 week

provided a much more robust decrease in TRPC6 expression. The divergent effects of TNF-α stimulation on TRPC3/6 may indicate that the ratio of their expression is important in facilitating the inflammatory response. A similar observation has been shown in human erythroid precursor cells in response to erythropoietin resulting in a greater intracellular calcium response (Hirschler-Laszkiewicz *et al.*, 2009).

3.5.3 Effects of IL-13 and TGF-β on Ca^{2+} handling protein expression

Previous work in our lab discovered an interesting interaction between IL-13 and TGF-β on TRPC6 gene expression (Karner, 2010). Stimulation with IL-13 alone had no discernible effect and TGF-β alone caused a small but significant increase in expression however when the two were combined a large synergistic increase was observed. A large portion of the transient receptor potential family was tested in this way and only TRPC6 reacted in a synergistic manner. At the time no suitable antibodies were available to investigate protein expression and only limited work was carried out on the functional consequences of this so the present chapter built upon the foundation gained previously.

3.5.3.1 TRPC6

IL-13 treatment did not affect TRPC6 protein expression reflecting the previous work on mRNA, also in accordance with this TGF-β resulted in a robust up-regulation of protein. The synergistic effect of the two in combination however was not observed possibly indicating a pre-translational level of control to prevent excessive Ca^{2+} entry into the cell. While analysing the western blots an interesting observation was made, one of the TRPC6 splice variants detected were affected differentially to the rest. The more recently described 70kDa TRPC6 splice (accession number: AK027769) is enhanced to a greater degree than the other three higher weight splices around 100kDa. The 70kDa splice is truncated from the N-terminal coil-coiled domain onwards and therefore missing the ankyrin repeat segment too. The functional relevance of any of the TRPC6 splice variants have yet to be established so the differential regulation of them by inflammatory cytokines can only be speculated. Truncation studies involving an N-terminal deletion of the ankyrin repeats up to the coil coiled domain in TRPC3 resulted in a trafficking defect (Wedel *et al.*, 2003). Furthermore truncation of TRPC6 at the N-terminal ankyrin repeat domain also resulted in faulty trafficking to the plasma membrane (Hofmann *et al.*, 2002). Upon co-expression with native TRPC6 or TRPC3 plasma membrane trafficking was restored. It is possible that the shorter 70kDa splice interferes with trafficking to the membrane and a reduction of the functional channel present.

3.5.3.2 SERCA2 and TRPC3

SERCA2 protein expression was enhanced by TGF-β expression for up to 72 hours of stimulation at which point it returned to control levels. IL-13 had no effect on its own or in addition to TGF-β on SERCA2 or

altering TRPC3 protein expression as had previously been shown for mRNA levels (Matsumoto *et al.*, 2012). One might have expected SERCA2 to be reduced as TGF-β is raised in asthmatics (Redington *et al.*, 1997) and the expression of SERCA2 is negatively correlated with asthma severity (Mahn *et al.*, 2009). This has been shown previously where SERCA2 was reduced by TGF-β which persisted even after the stimulus had been removed (Ojo, 2011). Even though the protein expression data here is in direct contrast to that of Ojo, it is consistent with the elevated mRNA levels observed following 6 hours TGF-β stimulation.

TRPC3 is also potentially increased in a similar manner to TRPC6 following TGF-β stimulation so the increase in SERCA2 expression may be to counteract an increase in Ca^{2+} leak into the cell. However the bands being analysed still need to be confirmed. There are in fact two splice variants of TRPC3 known, named one and two weighing 105 and 97kDa respectively. Therefore the two bands could both be TRPC3 and differentially regulated to allow finer control of function, however this is purely speculative and the other band could be due to protein modifications such as glycosylation or simply non-specific binding.

3.5.4 TGF-β signalling

The importance of TGF-β signalling in maintaining airway remodelling has been shown in a murine model of asthma (McMillan *et al.*, 2005). Therapeutic administration of anti-TGF-β Ab uncoupled the inflammatory and remodelling processes by reducing peribronchiolar ECM deposition, ASM proliferation and mucus production without affecting established inflammation and cytokine production. Evidence towards active TGF-β signalling is also put forward as phospho-Smad2 expression is decreased and Smad7 increased in lung sections. Over-expressing Smad2 in airway epithelium in a house dust mite model of asthma increased airway hyperreactivity, smooth muscle hyperplasia and subepithelial collagen deposition (Gregory *et al.*, 2010). The classic signalling pathway downstream of TGF-β activation is Smad2/3 activation so coupled with the previous evidence it was hypothesised that TGF-β was mediating its effects via these two secondary messengers.

Short interfering siRNAs targeted against Smad2 and Smad3 were designed and synthesised to address this aim using a previously tested algorithm as shown in section 2.6.1. Following transfection via electroporation protein levels were measured by western immunoblot and of the pool of three siRNAs designed the one achieving the best knockdown was selected for further use. In the case of Smad2 a further pool of three had to be designed as none of the original set produced a satisfactory reduction in protein.

By using these siRNAs alone and in combination it was shown that the increase in TRPC6 induced by TGF-β stimulation was dependent upon both Smad2 and Smad3. It has been shown in podocytes that TGF-β can upregulate TRPC6 protein expression and phosphorylation possibly via a Smad3-ERK-NF-κB pathway to increase cytosolic calcium and apoptosis (Yu *et al.*, 2010) which fits the data described here. Smad7 belongs to a sub-family of Smads known as the "anti-Smads" because of its ability to interfere with TGF-β signalling. It has been shown to integrate the input of IFN-γ and TGF-β signalling by binding to the TGF-β receptor complex and inhibiting its interaction and phosphorylation of Smad3 (Ulloa *et al.*, 1999). Although elevated IFN-γ isn't a typical feature of asthma it may be present in low levels contributing to AHR (Kumar *et al.*, 2006) and serves as an indicator on how a single cytokine's signalling cascade is integrated with others. The results here must be interpreted with caution as the cytokine storm present in asthma involves a plethora of mediators in which similar interactions are likely to take place.

On the same data set Smad2 and Smad3 protein levels were also probed to ensure the transfections worked (Figs. 3.4.3-2 and 3.4.3-3). In addition to the positive transfection and knock down ($p<0.001$ for both) an interesting observation was made that TGF-β stimulation significantly attenuates Smad3 protein levels ($p<0.001$) and only subtly reduced Smad2 (not significant). Although phosphorylation levels were not measured the 75% reduction in total Smad3 protein levels could be indicative of a negative feedback loop to prevent excessive signalling and changes in gene transcription. A full time course of Smad3 expression in response to IL-13, TGF-β and the combination of both showed IL-13 alone had no affect at all, TGF-β markedly reduced Smad3 expression as did the combination at each time point from 24 hours. The summation of these results presents an interesting point; TGF-β increases TRPC6 expression in a Smad3 dependent manner as shown by reducing Smad3 protein by siRNA. However TGF-β stimulation also reduces Smad3 expression to an equivalent level as the siRNA from 24 hours post stimulation but still increases TRPC6 expression for up to 7 days. Thus suggesting that Smad3 is important for the initial increase in TRPC6 expression but not for its maintenance. To confirm the involvement of Smad3 a constitutively active form of it could be used to ensure that the changes in TRPC6 are dependent upon it alone and not by other mediators that are activated by TGF-β (Funaba *et al.*, 2000).

The role of Smad4 was not evaluated in the present study but its role in TGF-β signalling should not be overlooked. It has been shown that TGF-β activation of TβRI induces heteromeric signalling complexes of Smad2/3/4 which translocate to the nucleus and is required for efficient signal transduction (Nakao *et al.*, 1997). There are no strong links with it to the asthmatic phenotype in the literature yet and it was shown to not be involved in TGF-β induced ASM proliferation (Xie *et al.*, 2007). However it may regulate mucus

hypersecretion (Harrop *et al.*, 2013) and due to its intracellular interactions with Smads2/3 it may play a subtle modulatory role which could be investigated using the siRNA methods outlined here.

3.5.5 Effects of IL-13 and TGF-β on intracellular Ca^{2+} transients

After one week of treatment with TGF-β and IL-13 there was no observed change in SERCA2 protein expression however the peak calcium release to bradykinin was diminished (p=0.0068, Fig. 3.4.4-1 upper) which is believed to be an indicator of store content. The pleiotropic actions of TGF-β lead to a wide range of possibilities underlying this discrepancy, such as altered expression of phospholipase C. The issue could be addressed by trying a range of agonists depleting store content to see whether this is a bradykinin specific effect or a true indication of store content. Another possibility is that only the function of SERCA2 is altered and not its expression which is backed up by the reduced decay rate measuring the rate of recovery back to baseline calcium levels (p=0.002, Fig. 3.4.4-1 lower) and is largely dependent upon SERCA2 function. There is evidence that CaMKII and ORMDL3 may regulate SERCA2 activity in the ASM which could be the targets of TGF-β (Cantero-Recasens *et al.*, 2010; Sathish *et al.*, 2009).

The decrease in calcium ion entry elicited by FFA following TGF-β and IL-13 treatment was also contrary to the initial hypothesis. It has been shown that FFA can activate TRPC6 so following on from the large increase in protein expression observed one would expect an increase in cytosolic calcium. It has been shown that the expression level of TRPC3 can influence its function and regulation in distinct ways (Vazquez *et al.*, 2003). Over-expressing TRPC3 to very high levels in this study ablated its ability to perform SOCE while retaining its sensitivity to phospholipase C activation. It has been hypothesised that increasing the expression of TRPC3/6/7 may titrate one or more of the regulatory subunits such as Orai1which is essential for SOCE (Liao *et al.*, 2007). Another potential explanation could lie with the 70kDa TRPC6 splice variant which increases further than the functional ≈100kDa splices. As this splice increases it could be incorporated into more tetramers and as it is missing a large portion of the N-terminus including the ankyrin repeat domain (ARD) necessary for certain subunit assembly and trafficking to the membrane the channel may not function correctly.

3.5.5.1 The role of TRPC6

The extent of the calcium ion current elicited by FFA going through TRPC6 needed to be addressed due to the high promiscuity of the drug. Knocking down TRPC6 with siRNA showed that a small but significant portion of the rise in cytosolic calcium was dependent upon the channel's expression however it was not the entire story. Knock down of Smad2 and Smad3 prevented the TGF-β induced effect completely showing that although TRPC6 is not the sole endpoint of the signalling cascade, Smad2/3 are critical for this

transduction. As shown previously, administration of TRPC3 siRNA completely ablated the increased response to FFA following TNF-α treatment and only partly attenuated the increased SOCE response. It would be interesting to investigate the effect TGF-β treatment has on SOCE and what role both TRPC6 and TRPC3 play by using the siRNAs. It is likely that they are implicated however not essential in the SOCE response in unstimulated cells however from the observed data it could be hypothesised that they are altered with cytokine treatment enhancing the response. Measuring changes in Orai1 and STIM1 protein production would also be necessary to elucidate the exact control mechanisms ASM cells exhibit in the face of inflammation.

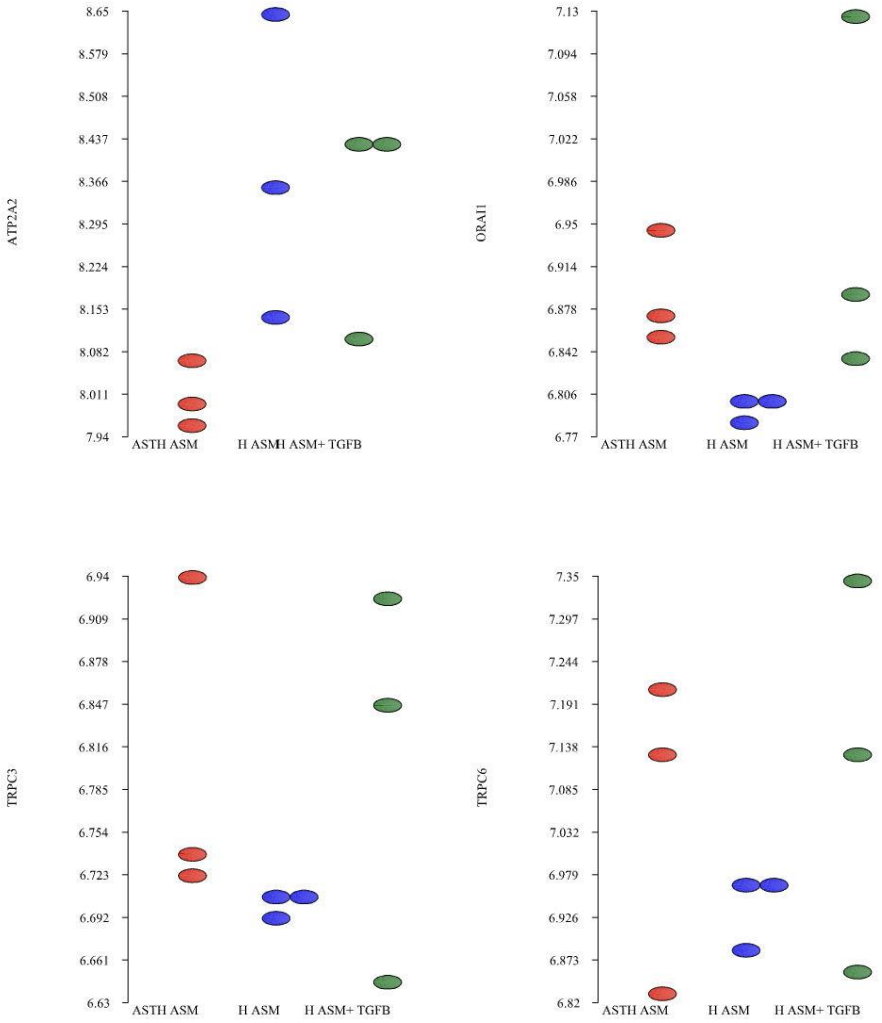
The role of TRPC6 in agonist dependent calcium entry following TGF-β treatment was investigated by reducing its expression with siRNA and stimulating with bradykinin. The reduction in TRPC6 had no effect on the unstimulated cells however the reduction in calcium mobilisation observed after TGF-β treatment was blunted. The mechanism behind this could again be attributed to specific expression levels of TRPC6 being required for optimal function or a role of the 70kDa splice. The TGF-β stimulation increases the smaller splice variant which may inhibit receptor trafficking resulting in diminished calcium elevation to bradykinin. Upon TRPC6 RNA interference the 70kDa splice is reduced and therefore partially restoring the function. Bradykinin stimulation can lead to a biphasic calcium response indicative of both store release and calcium entry of which the TRPC channels would play a role in the latter. This was not obviously present in the traces attained but could be still occurring and influencing the rate of recovery via direct store filling. Repeating the experiment in the absence of calcium in the perfusate would remove the compounding effect of SOCE. The roles of Smad2/3 were investigated but no inference could be made due to the positive control not working. An interesting additional experiment would be to look at contraction and the possible role the TRPC proteins and splices play, linking it to the altered calcium transients.

Recently further progress has been made in developing a specific TRPC6 agonist owing to its involvement in hypoxia induced vasoconstriction (HPV) and idiopathic pulmonary arterial hypertension (PAH) (Yu *et al.*, 2009). An agonist (8009-5364) has shown 2.5 fold selectivity for OAG induced TRPC6 over TRPC3 Ca^{2+} influx which is still only marginal but may shed some more light on the current mechanisms explored (Urban *et al.*, 2012).

3.5.6 Methylation

The fact that ASM cells derived from asthmatics retain some of their phenotypes and characteristics in culture after removal from the asthmatic environment raises some interesting points. One of which is the potential role DNA methylation may play in sustaining a modulated state in ASM cells driving remodelling

after the inflammation has resolved. Therefore cultured cells derived from asthmatic patients were compared to those from healthy patients to see if any of the genes of interest had a correlating methylation pattern. Following evidence that TGF-β induces a reduction in SERCA2 expression which is maintained after its removal (Ojo, 2011), the effect of TGF-β stimulation on healthy cells was also observed (Fig. 3.5.6-1).



3.5.6-1 An Illumina® 450K methylation array kit was used in conjunction with Dr Lavender's group to analyse CpG methylation in three ASM cultures derived from moderate asthmatics (red circles), "healthy" patients (blue circles) and "healthy" patients stimulated with 10ng.ml^{-1} TGF-β for 6 hours. Y-axis is % methylation at the cytosine.

Lower levels of methylation at the CpG sites correlate with higher levels of gene expression and it is considered that a mean fold change of approximately 1.5 is considered to be the minimum for relevant expression changes (Dr Lavender) of which none of the genes above met.

The SERCA2 gene ATP2A2 expressed no sign of change following TGF-β treatment fitting with the protein expression data at the earliest time point. Contrary to the work by (Mahn *et al.*, 2009) there was a subtle trend of reduced methylation in the asthmatic derived cells in this gene compared to the healthy cells which could result in increased expression. The TRPC3/6 genes bore no correlation regarding methylation pattern between the three groups and therefore the changes observed in this chapter are likely to be controlled by other mechanisms. Interestingly ORAI1 exhibited increased methylation in both the TGF-β treated healthy cells and the asthmatic derived cells compared to the healthy untreated cells. Although the protein expression levels of Orai1 following TGF-β treatment weren't in the scope of the current investigation there is some evidence that it may be increased in rat bronchial smooth muscle cells possibly contributing to increased SOCE (Gao *et al.*, 2013). The protein expression data presented here would seem to back up this finding as increased TRPC3 and TRPC6 expression could also aid the increased SOCE observed. The methylation data would seem to contradict these findings however the fold changes observed are minimal and as such are likely to have little impact on gene expression. Furthermore only three cell lines were investigated resulting in very low power.

3.5.7 A role for TRPA1

Preliminary data to some potentially very interesting work was performed in collaboration with Dr Andersson with the assistance of Dr Shaifta and Dr Prieto-Lloret. TRPA1 has been shown to be expressed on ASM and contributing to non-neurogenic inflammation in the airways (Nassini *et al.*, 2012). It could therefore be contributing to this via the ASM bundle. We therefore sought to discover whether it is functionally active and capable of causing contraction. Stimulation with its agonist AITC resulted in a robust dose dependent increase in peak intracellular calcium concentration as measured by fura-2. The concentrations at which rises in calcium were observed were low enough to be reasonably sure they were mediated by TRPA1. A TRPA1 antagonist AP18 (data not shown) resulted in a right-ward shift of the curve further backing up the interpretation.

It was then sought to find out whether the elicited rise in calcium would result in contraction in murine primary bronchioles and if this was dependent upon TRPA1 expression by using a homozygous knockout mouse. No contraction was observed in either genotype mice contrary to our hypothesis. TRPA1 is expressed in the mouse smooth muscle and epithelium (Nassini *et al.*, 2012) however its function has yet to be tested. A lack of contractile response does not negate the fact that the calcium elevation may be important for controlling other ASM functions in health and disease. TRPA1 can be activated by a wide range of endogenous by-products of oxidative and nitrative stress (Andersson *et al.*, 2008; Materazzi *et al.*,

2008; Sawada *et al.*, 2008) and there are highly elevated levels of oxidative stress in asthma which is implicated with exacerbations (Mak *et al.*, 2013). It is therefore plausible that TRPA1 activity on ASM is altered in asthma and could contribute to the altered phenotype in ways other than contraction such as the synthetic phenotype. Future studies investigating the expression and function of TRPA1 in health and asthma on ASM could provide new insights into the pathogenesis and potentially provide a novel therapeutic target.

3.5.8 Caveats

A major drawback to this study as already mentioned is the lack of pharmacological tools for TRPC investigation. FFA was chosen as a TRPC6 agonist based on findings in ciPod and HEK293 cells that it signalled independently to TRPC3 (Foster *et al.*, 2009). This is clearly not the case here as using a TRPC3 siRNA completely abolished the increased FFA response to TNF-α whereas the TRPC6 siRNA only partially inhibited the decreased response to TGF-β.

A second caveat that needs addressing is to better distinguish between agonist stimulated calcium store release and the so called receptor operated calcium entry. Stimulation of the cells with bradykinin would result in both. Therefore to determine the source of the rise in calcium and hence the involvement of TRPC channels if it is coming from the extracellular space the protocol needs to be refined to include the use of receptor antagonists and zero extracellular calcium.

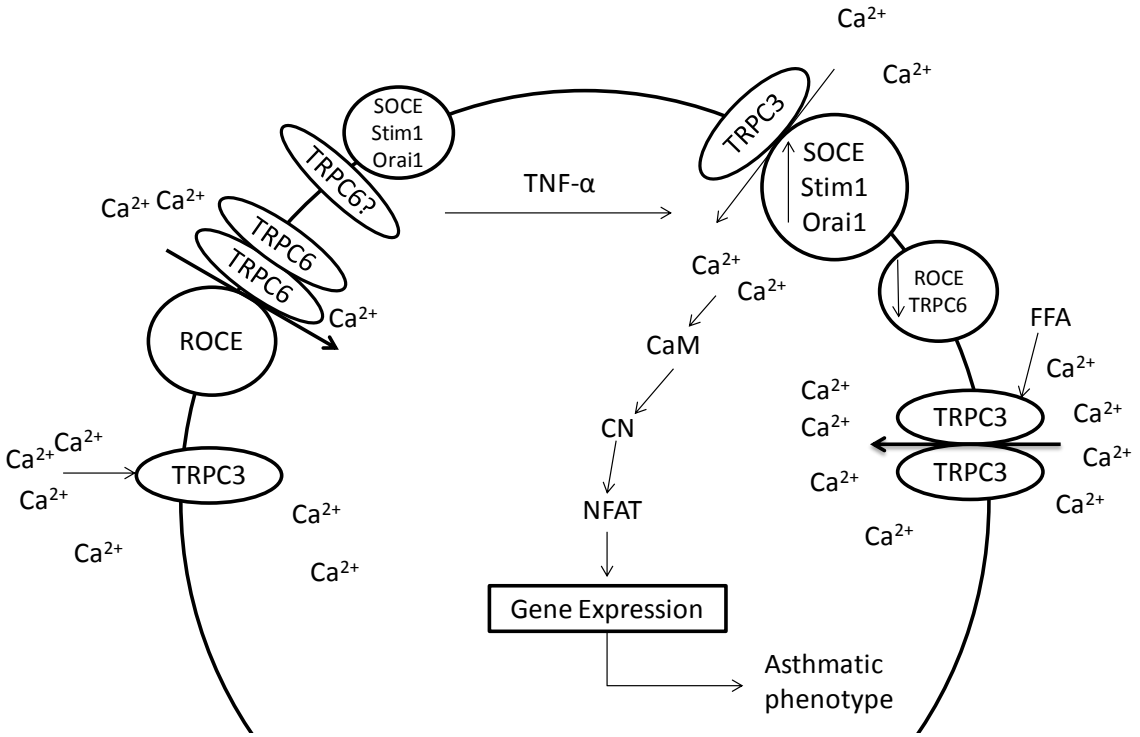
3.5.9 Conclusion

There is currently only a limited interest in TRPC6 as a target for pulmonary disease shown by the single patent of a TRPC6 antagonist by Sanofi-Aventis for use in COPD (Preti *et al.*, 2012). The IC_{50} value quoted of 12nM is promising however specificity of action and further understanding of the role of the TRPC proteins in the pathophysiology of asthma are very important if a new therapeutic target is achieved. TRPA1 antagonism is an even more promising and further developed target in respiratory research. There are compounds in preclinical trials for asthma (Preti *et al.*, 2012) which primarily focussed on their antitussive and neurogenic inflammatory effects. However they may be of additional benefit in restoring ASM calcium handling.

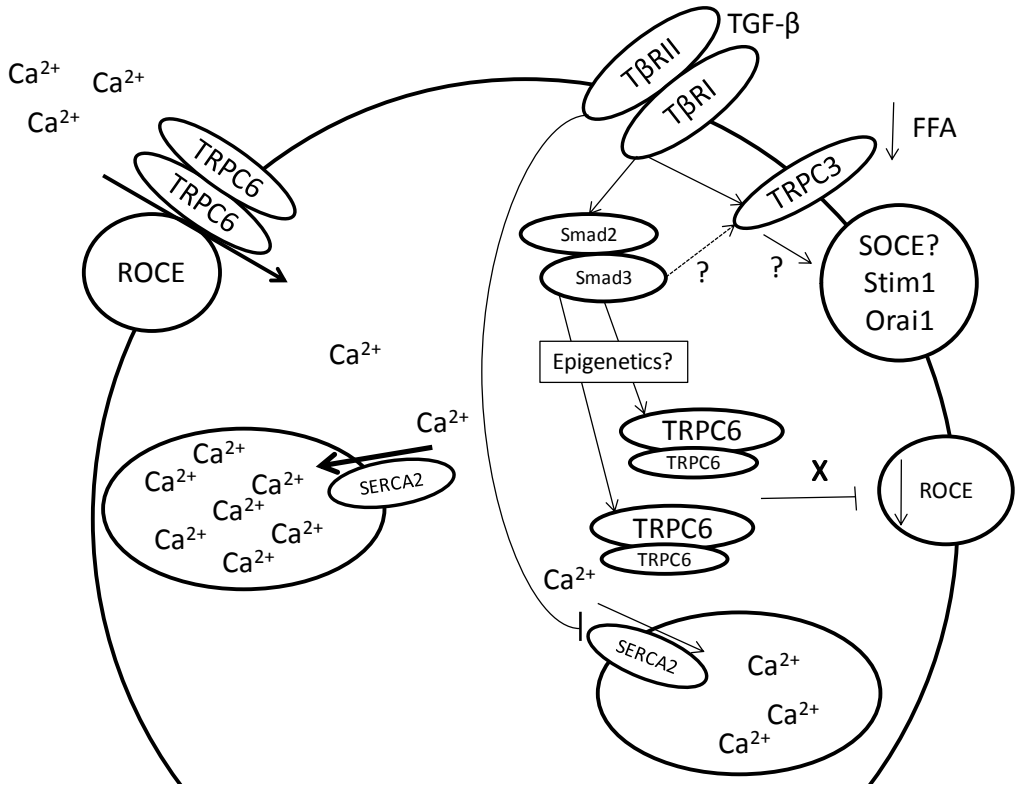
It is clear that the physiology of TRPC channels is very diverse and regulated by numerous factors such as expression levels and subunit specific tetramerisation. The experiments here show that cytokines implicated in asthma alter the protein expression of both TRPC3 and TRPC6 in ASM cells. The observed changes are to such a degree that the dynamics of calcium handling within the cell are altered. The delicate intricacies of

spatial and temporal specific calcium oscillations are largely lost in cultured ASM cells and it is this that integrates the wide variety of cellular stimulations into a functional response. It is important to try and tie the altered response observed in these experiments to the altered asthmatic phenotype of ASM cells and the signalling pathway in between to generate new therapeutic targets.

3.6 Summary of results



3.5.9-1 A potential mechanism of the ASM response to long-term TNF-α stimulation based on current findings. Upon stimulation with TNF-α there is a switch from ROCE to SOCE (White *et al.*, 2006). This is accompanied by a reduction in TRPC6 aiding the ROCE and an increase in TRPC3 aiding SOCE and increasing the basal leak of calcium into the cell. TRPC6 has been shown to interact with Orai1 (Liao *et al.*, 2007) and may confer to low levels of SOCE which are subsequently enhanced by TNF-α and increasing TRPC3 expression. The summation of these responses could result in the enhanced bronchoconstriction (Thomas *et al.*, 1995) and synthetic phenotype (Ghaffar *et al.*, 1999) possibly via NFAT (Mancarella *et al.*, 2013).



3.5.9-2 A potential mechanism of the ASM response to long-term TGF-β stimulation based on current findings. TGF-β increases TRPC6 expression in a non-uniform manner, preferentially up-regulating the smaller 70kDa splice which may exhibit impaired trafficking to the membrane. This process may involve epigenetic modifications as it is dependent upon Smad2/3 signalling to initiate but not to maintain. Calcium mobilisation to bradykinin was found to be independent to TRPC6 in healthy cells however upon stimulation with TGF-β a marked reduction was observed which was dependent partly upon TRPC6 expression. A reduction in the response to FFA was also observed indicating the enhanced smaller splice variant maybe reducing TRPC3 expression at the membrane also. TGF-β stimulation did not affect SERCA2 expression but did reduce its function which would further contribute to the smaller agonist induced calcium mobilisation.

3.6.1 How data fits with current literature

Not all of the data in this chapter concurred with the current literature. Due to the poor testing of TRPC antibodies by many and differing time-points investigated the work here presents a good addition to the field.

Finding	In accordance?	Reference
TNF-α increase TRPC3 and basal Ca^{2+} leak and switch from ROCE to SOCE.	Yes	White *et al*., 2006
TNF-α decreased agonist induced SR Ca^{2+} mobilisation.	No/Yes	No, later time-point measured White *et al*., 2006 Yes, Sathish *et al*., 2009 observed decreased SERCA2 expression.
TNF-α doesn't affect SERCA2 protein expression.	No	Later time-point measured Sathish *et al*., 2009
TGF-β increases SERCA2 expression up to 72 hours stimulation.	No	Ojo, Thesis, 2011
IL-13 doesn't affect TRPC3 protein expression.	No	Matsumoto *et al*., 2012

Chapter 4 Investigating the role of SERCA2 in an *in vivo* model of acute asthma

4.1 Introduction

4.1.1 A role for SERCA2 in asthma?

There is strong *in vitro* evidence for a role of SERCA2 down regulation and perturbed calcium handling in ASM cells contributing to an asthmatic phenotype (Mahn *et al.*, 2009; Mahn *et al.*, 2010). The paper demonstrates that SERCA2 protein expression is diminished in cultured ASM cells derived from asthmatics compared to those from a non-asthmatic population. Furthermore the degree of SERCA2 reduction correlated with disease severity and an asthmatic phenotype could be induced in healthy cells simply by reducing its expression with siRNA. The experiments highlighted the possible role of SERCA2 in the development or maintenance of the disease warranting further validation via other methods. This chapter sought to elucidate whether a reduction of SERCA2 *in vivo* could induce or exacerbate an asthmatic phenotype in a murine model of asthma.

4.1.2 Modelling asthma *in vivo*

Asthma is a heterogeneous disease, and common hallmarks of the disease include changes in lung function, specifically, airway hyperresponsiveness (AHR) to spasmogens, pulmonary inflammation and airway remodelling.

Due to these many facets of asthma it is difficult to investigate the disease as a whole; therefore numerous models have been developed to piece together information. Delineating intracellular signalling cascades and changes in protein expression are most effectively investigated *in vitro*, whereas *ex vivo* models are used for real-time functional and physiological measurements of ASM including contraction. Finally *in vivo* models investigate how the whole pulmonary and systemic system works as a unit and translates findings from the previous two approaches into a whole-organism disease setting (Wright *et al.*, 2012a).

There are numerous different techniques and models within each of these approaches which are tailored towards various end-points. A murine model has been chosen in this instance as the primary aim is to investigate the role of SERCA2 *in vivo* within the context of an asthmatic pathophysiology. Using a genomic knockout of SERCA2 is the best way to answer this aim as a straight comparison can be drawn displaying the impact of a lowered protein expression. Murine models of asthma are abundant and well established and a SERCA2 knockout line already exists.

4.1.3 An ovalbumin model of acute asthma

The mice used in this study were a generous gift from Dr Gary Shull (Cincinnati, USA) who used them to investigate cardiac failure (Periasamy *et al.*, 1999). They have a rather unorthodox background of a mixed 129/SVJ and Black Swiss of which there is no current data of their use within the context of asthma or allergy. A preliminary study was therefore of great importance as it has been shown before that various inbred mouse strains can respond significantly differently from one another following ovalbumin sensitisation and challenge (Ewart *et al.*, 2000). It takes at least ten generations to backcross onto another background such as the more conventional BALB/c or C57BL/6 which have a well characterised response to ovalbumin (Fernandez-Rodriguez *et al.*, 2010; Pitchford *et al.*, 2004; Riffo-Vasquez *et al.*, 2012). There was not enough time to perform this so instead a preliminary test was performed to ensure an allergic state could be induced in this strain which was achieved (Ojo, 2011). The mice are a heterozygous knockout as the homozygous mice die during embryonic development. SERCA2 protein expression is reduced to 63% ($p<0.05$) of the wild type expression in the heart (Periasamy *et al.*, 1999). The reduction in expression is comparable to that seen in moderate asthmatic tissue sections when compared to healthy sections, 67% (Mahn *et al.*, 2009). A homozygous knockout would have been useful as an extra comparison to better elucidate the role SERCA2 plays *in vivo.* However as the heterozygous expression closely matches the reduced human asthmatic expression this model is satisfactory.

As the strain of mouse being used was relatively uncharacterised in an asthmatic model setting, ovalbumin was chosen as the allergen. Recently more clinically relevant allergens such as house dust mite have been preferred as they offer a more tractable insight to the human disease (Cates *et al.*, 2004; Lloyd, 2007). The ovalbumin model has been used more extensively and has a proven robust response in the endpoints evaluated making it suitable for this investigation. An acute sensitisation protocol was employed initially to investigate changes in inflammatory cell and mediator infiltration into the airways and lung function parameters such as AHR or changes in compliance and resistance.

4.1.4 Measuring lung function

The first measurements of dynamic lung mechanics in mice were made in the late 1980's where pulmonary resistance and compliance was assessed in live, anaesthetised, tracheotomised mice (Martin *et al.*, 1988). Measuring lung mechanics in this state provides a high level of precision which comes at the expense of losing the natural state of the animal. The phenotyping uncertainty principle explains this trade-off and shows how no method is optimal for measuring lung mechanics and sacrifices have to be made at the investigator's discretion (Bates *et al.*, 2003). At the other end of the spectrum is whole body

plethysmography, a non-invasive technique which measures enhanced pause (Penh) to indicate airway sensitivity. There are clear benefits gained from using a non-terminal procedure such as investigating the early and late phase response in the same animal, a clinical feature of asthma which is too often forgotten in research (Evans *et al.*, 2012). The use of Penh from whole body plethysmography has come under scrutiny for not being representative of airway resistance (Zhang *et al.*, 2009) but advances such as using a pneumotachograph in conjunction (flow plethysmograph) have shown the waveform is not dominated by conditioning (Lomask, 2006). A bridge between the two extremes has been made in the form of using a balloon catheter in the pleural cavity to measure pleural pressure, an indicator of airway resistance, in unrestrained guinea-pigs (Meurs *et al.*, 2006).

4.1.5 Aims and hypotheses

The aim of this chapter was to investigate whether the enhanced asthmatic phenotype observed *in vitro* when reducing SERCA2 protein expression is translated *in vivo*. In order to investigate this aim four experimental groups were used; wild type sham sensitised mice, wild type ovalbumin sensitised mice, $SERCA2^{+/-}$ sham sensitised mice and $SERCA2^{+/-}$ ovalbumin sensitised mice. The parameters measured were leukocyte infiltration into and around the airways, cytokine infiltration to the airways, pulmonary morphology and total lung resistance, compliance and reactivity. I hypothesised that immunisation with ovalbumin would enhance eosinophilia with a concomitant increase in T_H2 cytokines, airway reactivity and resistance and decrease compliance in both wild type and $SERCA2^{+/-}$ mice. Furthermore, the reduction of SERCA2 in the knockout mice would enhance these changes in both the sham and ovalbumin immunised animals. Reducing SERCA2 with siRNA in healthy ASM cells resulted in an increase in eotaxin-1 production after IL-13 stimulation *in vitro* (Mahn *et al.*, 2009). Therefore an elevated level of eosinophils into the airways would be expected in the knockout ovalbumin sensitised mice compared to their wild type counterparts as eotaxin is a chemotactic agent for them. No direct relationship between reduced SERCA2 expression and an increase in ASM contractility has been observed in the literature to date although such a relationship is quite plausible. Contraction can be initiated by a rise in $[Ca^{2+}]_i$ resulting in a calmodulin dependent activation of myosin light chain kinase which phosphorylates MLC_{20} producing cross bridge cycling and muscle shortening. The 4:1 stoichiometry between calcium and calmodulin would mean that only a small rise in $[Ca^{2+}]_i$, caused by a reduction in SERCA2, could lead to a far greater response to contractile agonists (Mahn *et al.*, 2010). Changes in lung morphology were also investigated and I hypothesised that only a genotype and not a treatment effect would be observed. The duration of the challenge protocol employed was such that one would not expect ovalbumin driven remodelling. However

as the SERCA2$^{+/-}$ mice have had an altered calcium handling from birth enough time would have elapsed by termination to potentially see a thicker smooth muscle layer around the airways.

4.2 Methods

Atp2a2 heterozygous knockout Black Swiss/129SVJ mice were used as homozygous knockouts are embryonic lethal, they were a gift from Dr Gary Shull. They were generated as described in (Periasamy *et al.*, 1999). As predicted by Mendelian genomics, offspring were produced in an approximate 1:1 heterozygous to wild type ratio when breeding a wild type with a SERCA2$^{+/-}$ mouse. Male and female mice were used equally in each treatment group to minimise any gender bias as found in the preliminary study. All studies were carried out according to The Animals (Scientific Procedures) Act (1986) and local ethical approval from King's College London.

Animals were housed in cages according to their gender in environmentally controlled rooms with access to food and water *ad libitum* before and during the experimental period.

4.2.1 Genotyping wild type and SERCA2 heterozygous knockout mice

The knockout mice displayed no overt phenotypic difference to the wild type, therefore to differentiate between them the mice had to be genotyped. DNA from ear clippings is first isolated using the "DNeasy Blood and Tissue Kit" (QIAgen, Crawley) and 50ng of DNA together with "illustra Ready-To-Go™ RT-PCR Beads" (GE Healthcare Life Sciences) is incubated with 2pM of SERCA primer mix on ice. The primer mix was comprised of three primers outlined below:

Primers (Sigma):

	Sequence	Catalogue number
Primer 1	5'-CGGCCTTCTAGAATTGCCGGCTG-3'	SY100204245-052
Primer 2	5'-CTTACGAAAGATATACATGCTGCCAGCAG-3'	SY100204247-031
Primer 3	5'-GCATGCTCCAGACTCCCTTG-3'	SY100204241-063

The PCR master mix is amplicated using the following protocol:

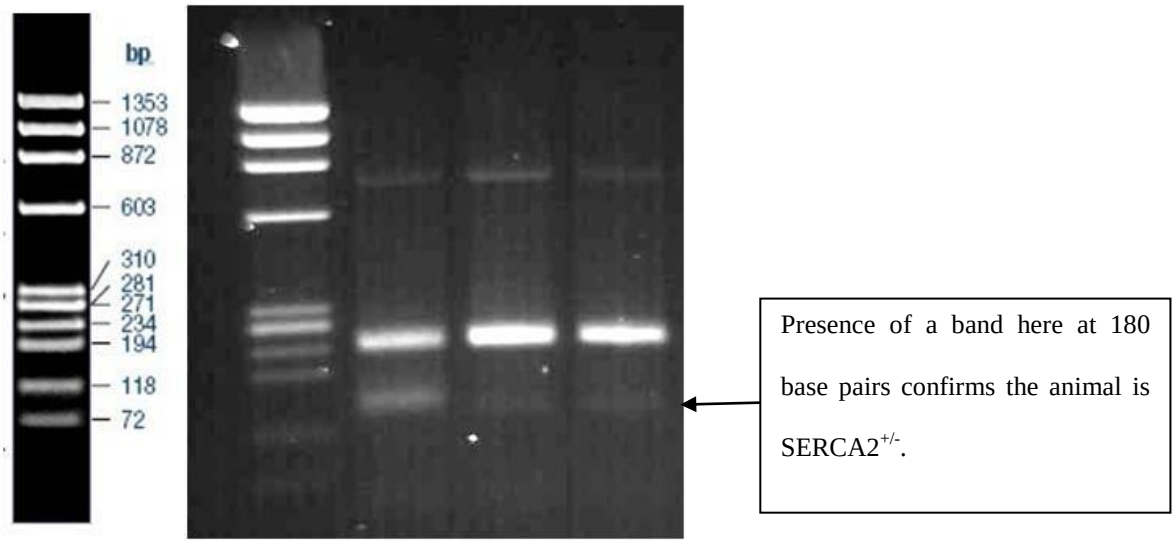
94°C for 3mins

95°C for 30secs
59°C for 30secs
72°C for 30secs
} x40

72°C for 5mins

4°C until gel loading

The PCR product is then run through a 2.5% agarose gel at 80V for 90 minutes and bands are compared to a molecular ladder (ΦX174 DNA/BsuRI HaeIII Marker, Fermantas). For wild type mice a band is visualised at 233 base pairs and for the heterozygous knockouts bands are present at 233 and 180 base pairs. The second band in the knockouts represents the insert primer 1 is targeted against which disrupts the gene expression.

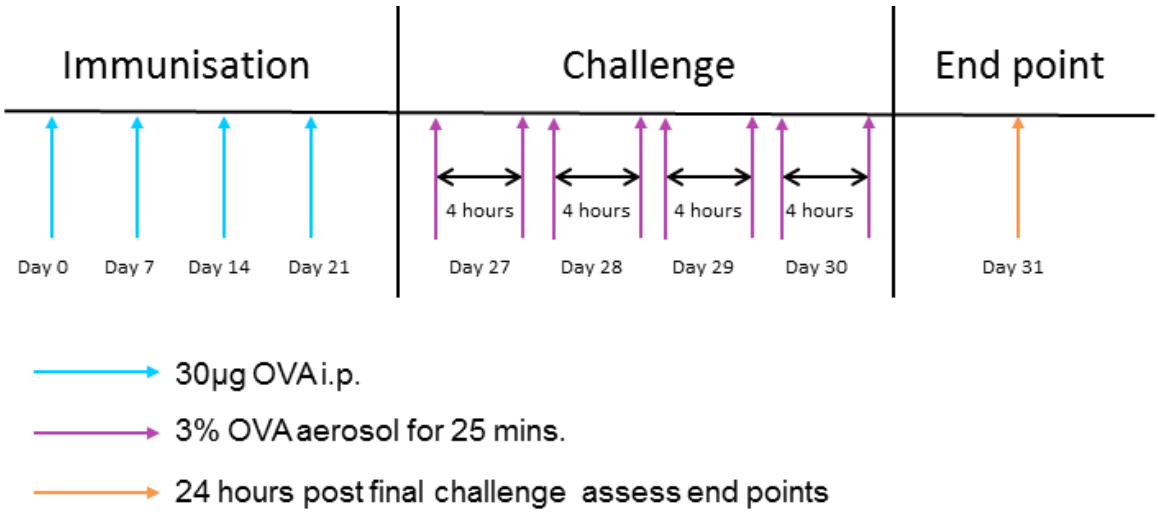


4.2.1-1 Gel showing the PCR products of SERCA2+/- (lane one) mice and wild type mice (lanes two and three). Briefly, tail clippings are digested and genomic DNA isolated using the QIAgen "Blood and Tissue Kit" then samples are run on a gel and bands are visualised using ethidium bromide.

4.2.2 Ovalbumin challenge

Mice were immunised on day 0, 7, 14 and 21 with 30µg/0.4ml chicken ovalbumin (OVA) intraperitoneally (i.p.) using aluminium hydroxide ($Al_2[OH]_3$) (Sanofi-Synthelabo, Sao Paolo, Brazil) in saline solution as the adjuvent. Mice were then exposed to aerosolised OVA ($30mg.ml^{-1}$) twice daily for 25 minutes on Days 27-30 in a specially designed 9L ventilated chamber allowing for free movement (see Fig. 4.2.2-1). Twenty-four hours after the final challenge mice were anaesthetised with $2g.kg^{-1}$ urethane i.p. (Sigma).

4.2.2-1 The 9 litre aerosolisation chamber the mice are kept in during the 25 minute ovalbumin exposure. Mice from different cages and genders are separated to prevent disruption.



4.2.3 Lung function

4.2.3.1 Respiratory lung mechanics

In this model the method of invasive tracheotomised measurements of lung function were employed (Pitchford *et al.*, 2004). Severity of bronchoconstriction was gauged by measuring changes in total lung resistance (RL) and dynamic compliance (Cdyn) in response to aerosolised methacholine. Variables such as the concentration of methacholine which caused a 200% increase in total lung resistance above saline challenge (RL PC200) and peak response (maximum RL) were recorded. Dynamic compliance was

obtained from continuous measurement of intrapleural pressure and volume during a normal breathing cycle (Ward *et al.*, 2010). It is measured as the slope between zero airflow at the end of inspiration and expiration on a volume pressure curve and is indicative of lung tissue and alveolar compliance. In health it is often similar to static compliance (the change in lung volume per unit change in distending pressure) but in disease it can fall. Total lung resistance describes the restriction of airflow from the atmosphere into the alveoli. Poiseuille's equation describes the factors affecting laminar flow showing that a halving of airway lumen radius will increase airway resistance 16 fold:

$$R = \frac{8ln}{\pi r^4}$$ Where R = Resistance, l = Length, r = Radius, n = Viscosity of fluid.

The values of total lung resistance (RL) and dynamic compliance (Cdyn) are derived by fitting the equation of motion to measurements of flow, pressure and volume under the assumption the chest wall presents little mechanical load compared to the lung (Irvin *et al.*, 2003):

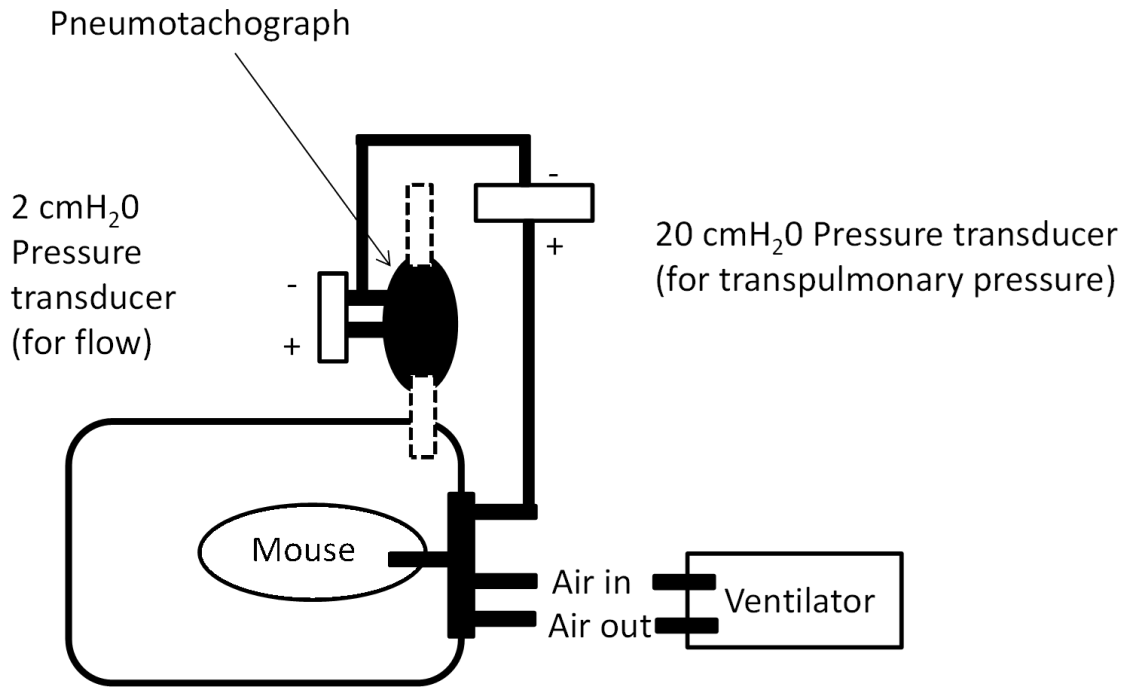
$$P = R\dot{V} + \frac{1}{C}V$$ Where P = Applied pressure, R = Lung resistance (RL), $\dot{V}$ = Flow of gas, C = Compliance (Cdyn), V = Lung volume relative to functional residual capacity.

An increase in RL is generally believed to relate to changes in the conducting airways and alterations in the lung periphery such as heterogeneous closing of the small airways and physical properties of the lung parenchyma. Decreases in dynamic compliance reflect primarily peripheral events such as airway de-recruitment (Irvin *et al.*, 2003).

Mice were laid supine and using blunt dissection techniques a tracheotomy was performed. The trachea was exposed, the ventral surface was sliced transversely and a cannula inserted and fixed in place with suture. After surgery, the mice were placed in a plethysmograph and the cannula was connected to a 4-way manifold with one port attached to a differential pressure transducer (± 20 cm H_2O; Validyne Engineering, Northridge, CA) for the measurement of mouth pressure (Pao). Two ports were connected to the inspiratory and expiratory ports of a volume-cycled ventilator (SAR-830A, model 963217; CWE, Norfolk, United Kingdom). Mice were ventilated at 150 breaths per minute with a tidal volume of 0.15mL to 0.2mL and a positive-end expiratory pressure of 2.5cm H_2O.

Changes in flow were determined with a Fleish pneumotachograph (size, 00000) connected to a side port of the chamber and measured with a differential pressure transducer (± 2 cm H_2O; model MP 45-14-871; Validyne Engineering). The flow was integrated to give a continuous recording of tidal volume.

Transpulmonary pressure was estimated as the difference between mouth pressure and the pressure measured at the proximal end of the pneumotachograph to the plethysmograph, inasmuch as the chest wall contributes little to the overall compliance of the respiratory system. Breath-by-breath recording of total lung resistance (RL; $cmH_2O/L/s$) and dynamic compliance (Cdyn; ml/cmH_2O) were calculated by an online respiratory analyser on a personal computer (Lung Function Recorder version 7; Mumed, London, United Kingdom), (Pitchford *et al.*, 2004).



4.2.3-1 A wiring diagram for the set up to measure lung function as described in the methods. The system takes advantage of the rise and fall of the rib cage to generate the positive and negative airflow signals.

4.2.3.2 Measurement of AHR to methacholine

AHR is determined in the clinic by a methacholine provocation test, administering increasing doses until a 20% fall in FEV_1 is achieved (Lotvall *et al.*, 1998). A similar method has been adopted previously in murine models and used here where sensitivity is measured by the concentration of methacholine required to increase total lung resistance by 200% above saline induced levels (RL PC200). Additionally the provocation concentration to cause a 50% decrease in Cdyn (Cdyn PC50) was used as a measure for sensitivity to change in dynamic compliance.

Aerosols of methacholine (1.56 – 25 mg/mL) were generated from an ultrasonic nebulizer (Aerocare) for a period of 8 seconds and administered directly to the lungs via the inspiratory port of the plethysmograph. RL was measured prior to administration of saline (0.9%) and following administration of saline or

methacholine. The maximum increase in RL to methacholine is expressed as a percentage of the values of RL obtained after saline challenge. The concentration of methacholine that induced a 200% increase in RL above postsaline RL was used as a measure of airway sensitivity (PC200) and maximum airway obstruction (Peak) was used as a measure of 'reactivity' (Pitchford *et al.*, 2004).

4.2.4 Cell counts

Bronchoalveolar lavage (BAL) was performed by administering 0.5ml of phosphate buffered saline (PBS) via the cannula into the lung and then retrieving the fluid into the syringe. This was repeated twice more with all three samples being pooled and kept on ice for cell counts.

4.2.4.1 Total cell count

Harvested BAL fluid is added in a one to one ratio to Turk's solution (a mixture of acetic acid and gentian violet) which lyses erythrocytes and stains leukocytes blue. The cells are then counted on a haemocytometer slide and multiplied by the two-fold dilution factor to give the total BAL cell count.

4.2.4.2 Differential cell count

The slides are prepared by adding 100µl of BAL fluid to a cytofunnel and spun onto a slide using a centrifuge set at 1000rpm for 1 minute. The slides are then stained by submerging them first in absolute methanol for 10 seconds, then in eosin and finally haemotoxin for 10 seconds each. Cover slips are added using DPX and the percentage of monocytes, neutrophils and eosinophils are calculated and combined with the total cell counts to give estimations of each cell type.

4.2.5 Cytokine determination

The concentration of cytokines within the BAL samples was determined by a multiplex fluorescent bead assay. The Luminex® bead assay works in a similar fashion to a combination of flow cytometry and sandwich ELISAs. Fluorescently dyed magnetic beads each with a distinct "spectral address" relevant to the analyte being measured were coated with a specific capture antibody. The assay can be multiplexed with detection of up to one hundred different molecules in a single well of a 96-well plate. The coupled beads bind with the sample containing all the biomarkers of interest and following a series of washes a biotinylated detection antibody was added to bind to the biomarker too. Streptavidin-phycoerythrin was finally added to serve as a fluorescent indicator. The beads were then classified to which biomarker they have a capture antibody against by using a red 635nm laser to illuminate the fluorescent dye. They were subsequently excited by a green 532nm laser to excite the phycoerythrin to generate a reporter signal of

which the mean fluorescence intensity is proportional to the amount of analyte bound. The system used in this thesis was from Millipore.

4.2.6 Tissue homogenisation

The anterior right lung lobe was tied off, removed and stored in at least 5 times the volume with one dimension <5mm in "RNAlater" for further analysis. The remaining lobes were stored either in 10% neutral buffered formalin (NBF) and used for histological staining. For the preliminary study lung and heart tissue were snap frozen in liquid nitrogen after removal. Tissue was subsequently pulverised while still frozen and added to 1ml working solution of lysis buffer and protease inhibitor. Due to the larger numbers involved in the main study heart and lung tissue was stored in RNA later at 4°C for 24 hours then frozen at -80°C. Once ready to be processed the tissue was thawed and added to 1ml working solution of lysis buffer, protease and phosphatase inhibitor as before and then lysed using the QIAgen TissueLyser II. Either way the homogenate was then centrifuged at 10,000*g* for 10 minutes at 4°C and supernatant removed, aliquoted and stored at -80°C for future analysis. Previous work has shown that this extraction technique can reduce the amount of protein phosphorylation detectable which should be taken into account when interpreting the results (Johnsen *et al.*, 2010).

4.2.7 Histology

Lungs were stored in 10% NBF for at least one week prior to histological analysis. The lobes were cut along their lateral axis and set into Paraplast embedded blocks and sectioned into 4µm thick slices using a microtome. The sections were removed of any Paraplast and rehydrated by treatment with xylene and decreasing concentrations of ethanol. For each stain four mice per group were used and representative images were taken blindly and included.

Hematoxylin and eosin (H&E) staining was used for general structure, Masson's trichrome for smooth muscle and collagen and periodic acid shift (PAS) for goblet cells. For the H&E stain slides were immersed in hematoxylin for 2 minutes followed by eosin for 2 minutes, washed in tap water and dehydrated with increasing concentrations of ethanol and cleared with xylene. Cover slips were then added with DPX mounting solution.

For the Masson's trichrome staining samples were re-fixed in Bouin's solution (75ml picric acid, 25ml of 40% formaldehyde and 5ml of glacial acetic acid) to improve staining quality for one hour. Slides were then washed in tap water and stained in Weigert's iron hematoxylin working solution for 10 minutes (equal parts solution A and B):

Weigert's Iron Hematoxylin Solution:

Stock solution A:	
Hematoxylin	1g
95% Alcohol	100ml
Stock solution B:	
29% Ferric chloride in water	4ml
Distilled water	95ml
Hydrochloric acid, concentrated	1ml

Slides were washed again in tap water and then distilled water and stained with Biebrich scarlet-acid fuchsin (Biebrich scarlet, 1% aqueous 90ml, acid fuchsin 1% aqueous 10ml and glacial acetic acid 1ml). Slides were washed again in distilled water and differentiated in in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes or until the collagen is no longer red. The slides are transferred directly to aniline blue solution and stained for 5-10 minutes, rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2-5 minutes. They are finally dehydrated as before and cover slips mounted with DPX.

4.3 Results

4.3.1 Characterisation of mice

	Wild type/Sham		SERCA2$^{+/-}$/Sham		Wild type/OVA		SERCA2$^{+/-}$/OVA	
	M	F	M	F	M	F	M	F
Age (Days)	89.1±7.4	89.1±7.4	89.1±7.4	89.1±7.4	89.1±7.4	105.1±6.5	89.1±7.4	89.1±7.4
Weight (g)	30.4±1.1	25.9±0.9	31.3±0.9	25.8±1.0	32.8±1.0	26.5±1.1	32.0±0.9	25.4±0.9
Baseline RL ($cmH_20/s/L$)	1566±199	1785±172	2032±159	1727±174	1621±186	1724±201	1664±172	1959±175
Baseline Cdyn (ml/cmH_20)	0.035±0.003	0.04±0.002	0.041±0.002	0.043±0.003	0.038±0.003	0.039±0.003	0.04±0.002	0.039±0.003

Table 4.3.1-1 A characterisation of the mice used in the following chapter, sub-divided into their experimental groups and analysed by a three way ANOVA with a Sidak post-hoc analysis to see if they differed prior to experimentation.

There were no significant differences in age between the groups.

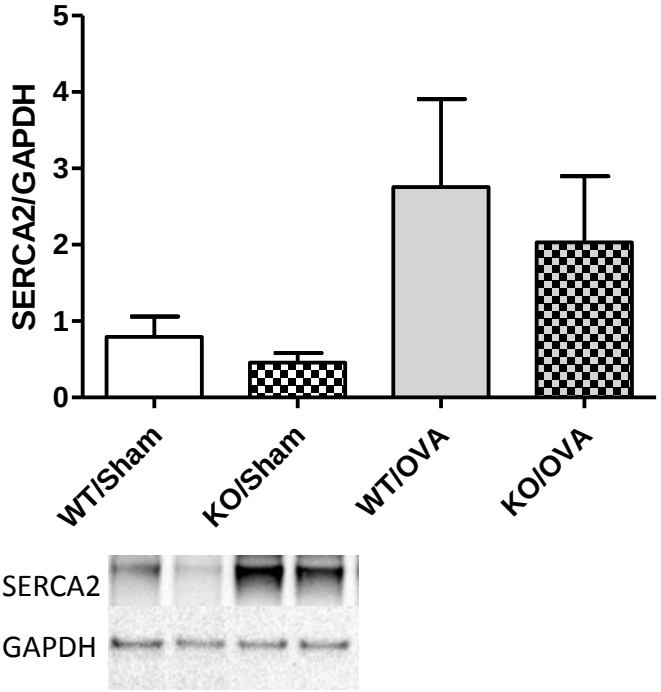
There was a significant difference of 5.74 ± 0.73g between the weights of male and female mice used in this chapter ($p<0.001$).

There were no significant differences in baseline RL between the groups.

There were no significant differences in baseline Cdyn between the groups.

4.3.2 Expression of calcium handling proteins in lung homogenate

Following genotyping as described in 4.2.1 it was important to establish whether the disruption of one of the Atp2a2 genes was enough to reduce protein expression in the lung as shown by Periasamy *et al.*, 1999 in the heart. The knockout of one allele was enough to significantly reduce SERCA2 protein expression in whole lung homogenate (Fig. 4.3.2-1). The reduction in SERCA2 protein expression in both the sham mice (0.80±0.27 compared to 0.46±0.13) and ovalbumin immunised mice (2.76±1.15 compared to 2.03±0.87) was similar to previously published results. A two way ANOVA showed that genotype significantly affected expression (p=0.03) however immunisation did not (p=0.13) and there was no interaction (p=0.34).



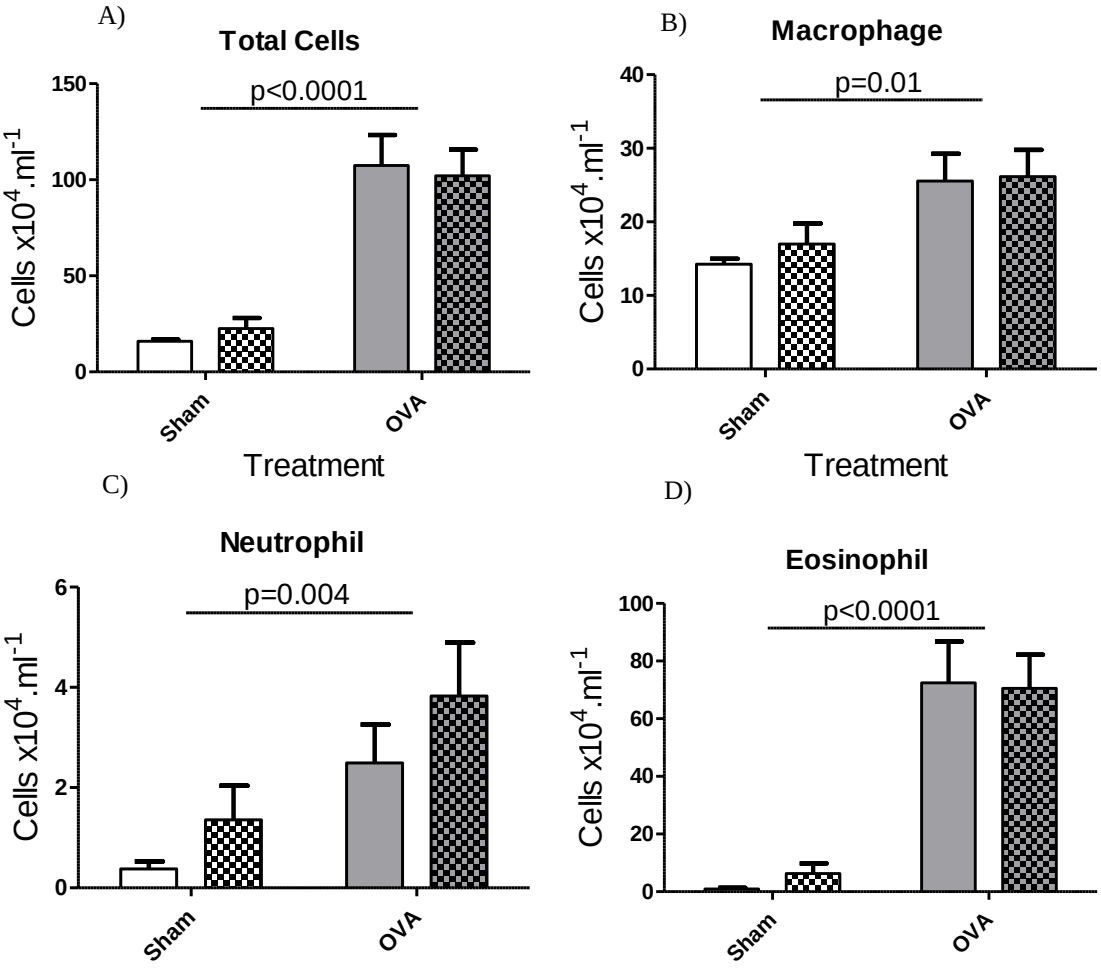
4.3.2-1 SERCA2 protein expression levels compared to GAPDH. Lanes from left to right indicate wild type/sham, Atp2a2$^{+/-}$/sham, wild type/OVA and Atp2a2$^{+/-}$/OVA. Bars represent mean±SEM, n=4.

The expression level of TRPC3 and TRPC6 could not be successfully quantified because of the lack of sensitivity/specificity of the commercially available antibodies used.

4.3.3 Effect of ovalbumin immunisation on inflammatory cells

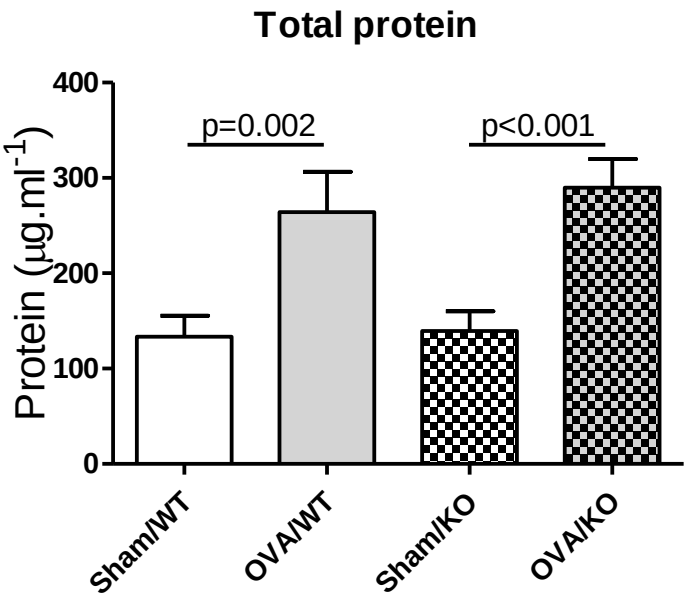
The extent of inflammatory cell infiltration into the lung was quantified in bronchoalveolar lavage fluid and subsequent enumeration using differential cell staining as described in section 4.2.4. The two questions being addressed were; did the immunisation increase the inflammatory infiltrate and did the genotype status affect the result? A two-way ANOVA was performed to test whether those two

independent variables impacted each cellular infiltrate endpoint. The immunisation status of the mice significantly affected the recruitment of the total cell count ($p<0.0001$), macrophages ($p=0.01$), neutrophils ($p=0.004$) and eosinophils ($p<0.0001$) (Fig. 4.3.3-1). As each inflammatory cell type tested was significantly enhanced in the airways of ovalbumin immunised mice it is clear the model was successful. The second independent variable being tested was the genotype of the mice and this had no significant effect on any of the four dependent variables tested; total cells ($p=0.95$), macrophages ($p=0.58$), neutrophils ($p=0.14$) and eosinophils ($p=0.85$). A trend was observed for increased neutrophil recruitment in SERCA2$^{+/-}$ mice compared to the wild type mice however no interaction was observed ($p=0.82$).



4.3.3-1 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge A) total inflammatory cells, B) macrophages, C) neutrophils and D) eosinophils were assessed in the BAL fluid. Data represents mean ± SEM of n=22-28 per group. No pattern bars represent wild type mice, chequered bars represent SERCA2$^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. A two way ANOVA was performed with a Sidak post hoc analysis. The P values illustrated represent the main effect of immunisation.

The total protein concentration in the BAL was used as an indication of plasma protein extravasation and oedema in the airways. Immunisation with ovalbumin resulted in a significant increase in plasma extravasation in both the wild type and SERCA2$^{+/-}$ mice ($p=0.002$ and respectively $p<0.001$, Fig. 4.3.3-2). A two way ANOVA showed a significant difference in mean values between immunisation status ($p<0.001$) but not genotype ($p=0.59$) with no significant interaction.

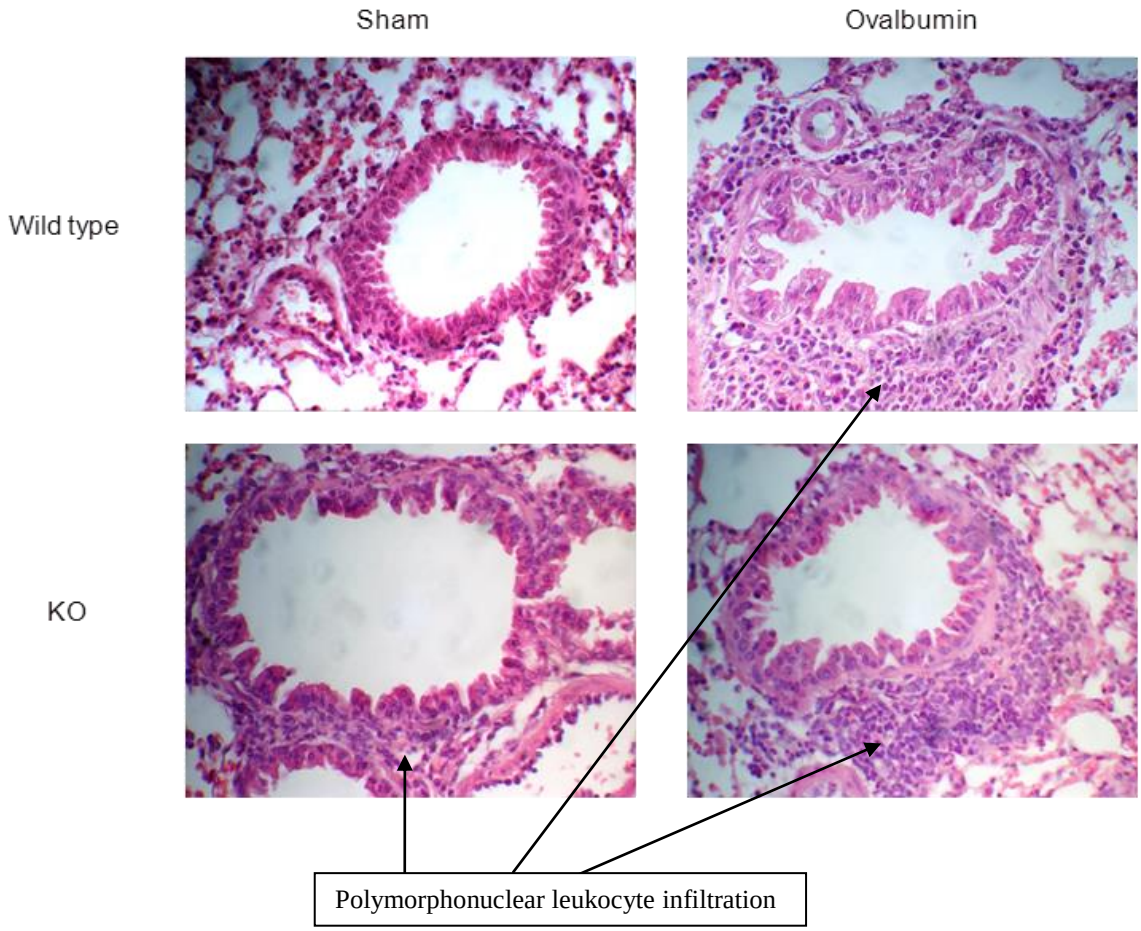


4.3.3-2 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge total protein levels were assessed in the BAL fluid. Data represents mean ± SEM of n=13-16 per group. No pattern bars represent wild type mice, chequered bars represent SERCA2$^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. A two way ANOVA was performed with a Sidak post hoc analysis. The P values illustrated represent the main effect of immunisation.

4.3.4 Effect of ovalbumin immunisation on H&E staining

To determine whether the cellular inflammatory response of the airways as shown by cells recovered from the BAL (above) was comparable to that in the surrounding lung parenchyma, tissue sections were taken and stained with H&E. The stain gives a good structural overview as the haematoxylin gives nuclear definition with a deep blue and the eosin provides a contrasting red counter-stain (Fig. 4.3.4-1).

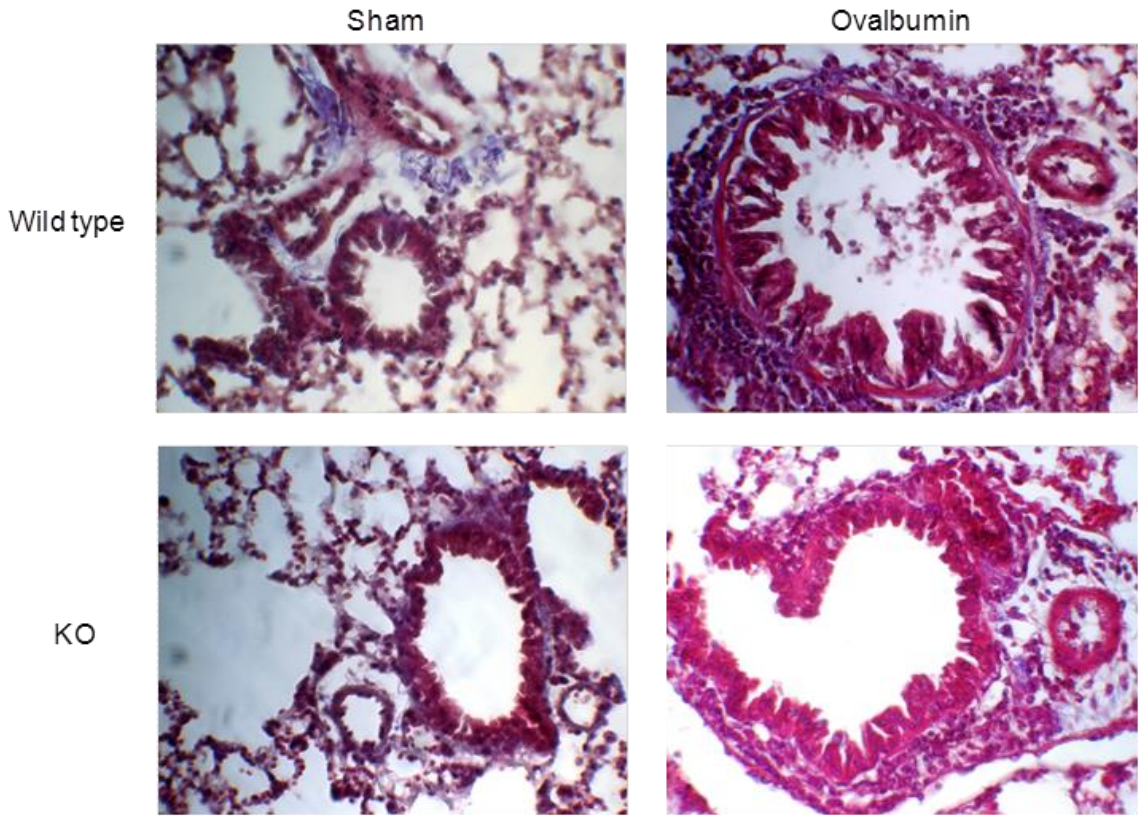
Reflective of the infiltrate in the airways following challenge to ovalbumin, there was also a large increase of inflammatory cells in the surrounding parenchyma. A notable increase of polymorphonuclear leukocytes was also observed in the sham/SERCA2$^{+/-}$ mice compared to the sham/wild type which was also present in the BAL.



4.3.4-1 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge lungs were removed and preserved in 10% formalin and 4µm paraffin embedded tissue sections were stained using H&E staining. Images are representative of their respective groups taken blindly from a sample of four mice per group on a x20 objective.

4.3.5 Effect of ovalbumin immunisation on smooth muscle and collagen deposition

Masson's trichrome stain was used to stain collagen (blue), smooth muscle (red) and nuclei (black) in the lung segments (Fig. 4.3.5-1). ASM was present in equal abundance surrounding the airways in all four treatment groups and in a similar morphology showing no signs of hyperplasia, thickening or migration. Levels of collagen also appeared to be unchanged by both immunisation status and genotype.

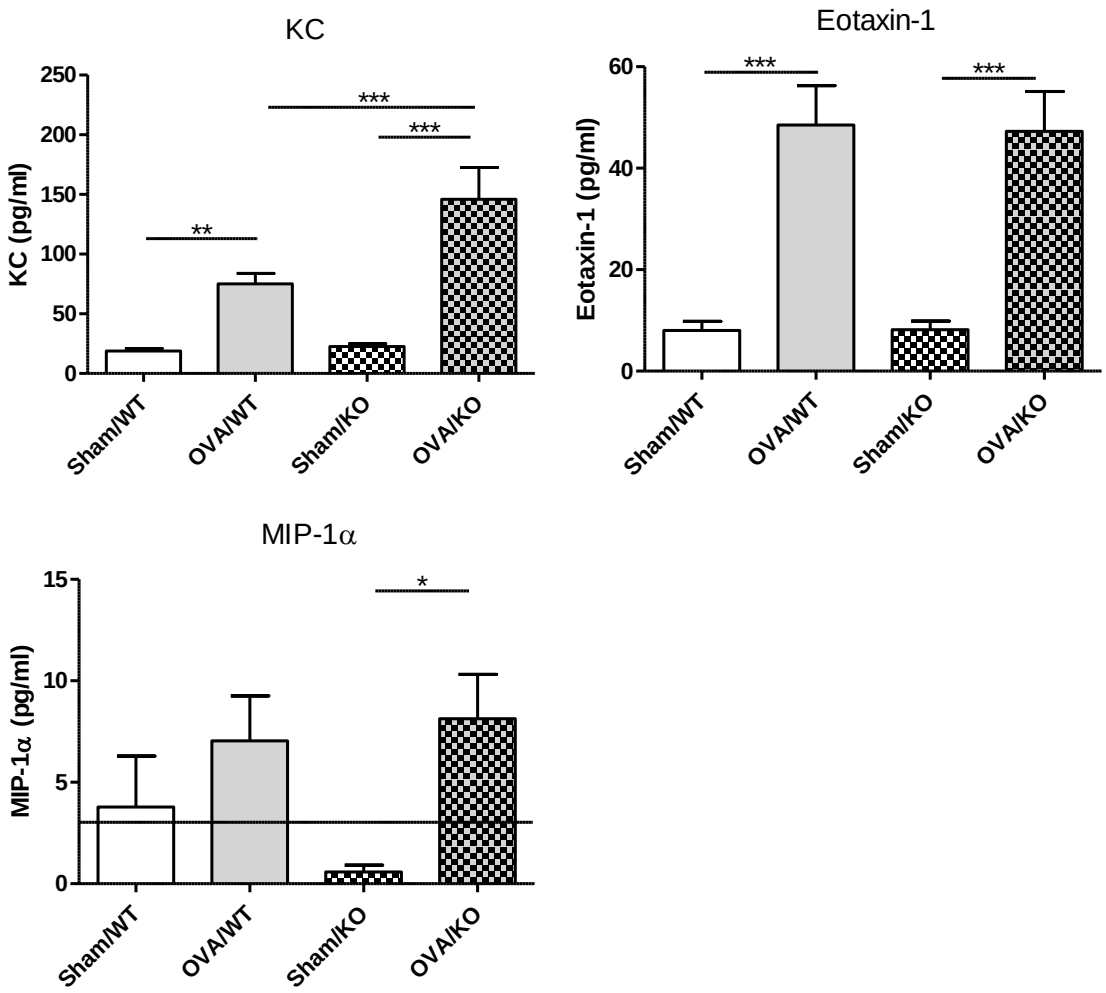


4.3.5-1: Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge lungs were removed and preserved in 10% formalin and 4µm paraffin embedded tissue sections were stained using Masson's trichrome for collagen. Images are representative of their respective groups taken blindly from a sample of four mice per group on a x20 objective.

4.3.6 Effect of ovalbumin immunisation on cytokine levels in the BAL

A luminex bead assay was performed measuring six proteins; KC, IL-13, eotaxin-1, IL-17, TNF-α and MIP-1α. The assay detected concentrations from 3.2 $pg.ml^{-}$, this level of detection was not achieved for the mean data for any of the groups measuring IL-17, TNF-α or IL-13 (Fig. 4.3.6-1).

There was a significant increase in the levels of KC, eotaxin-1 and MIP-1α following allergen challenge in the immunised mice ($p<0.001$, $p<0.001$ and $p=0.02$ respectively). Genotype only significantly impacted the levels of KC in the BAL ($p=0.008$) where an interaction was also observed between it and the immunisation status ($p=0.02$) which was further highlighted by the Sidak post-hoc test. The reduced SERCA2 expression in the knockout mice further enhanced the raised KC levels following immunisation ($p<0.001$). Eotaxin-1, akin to the eosinophil data observed no genotype affect ($p=0.92$).



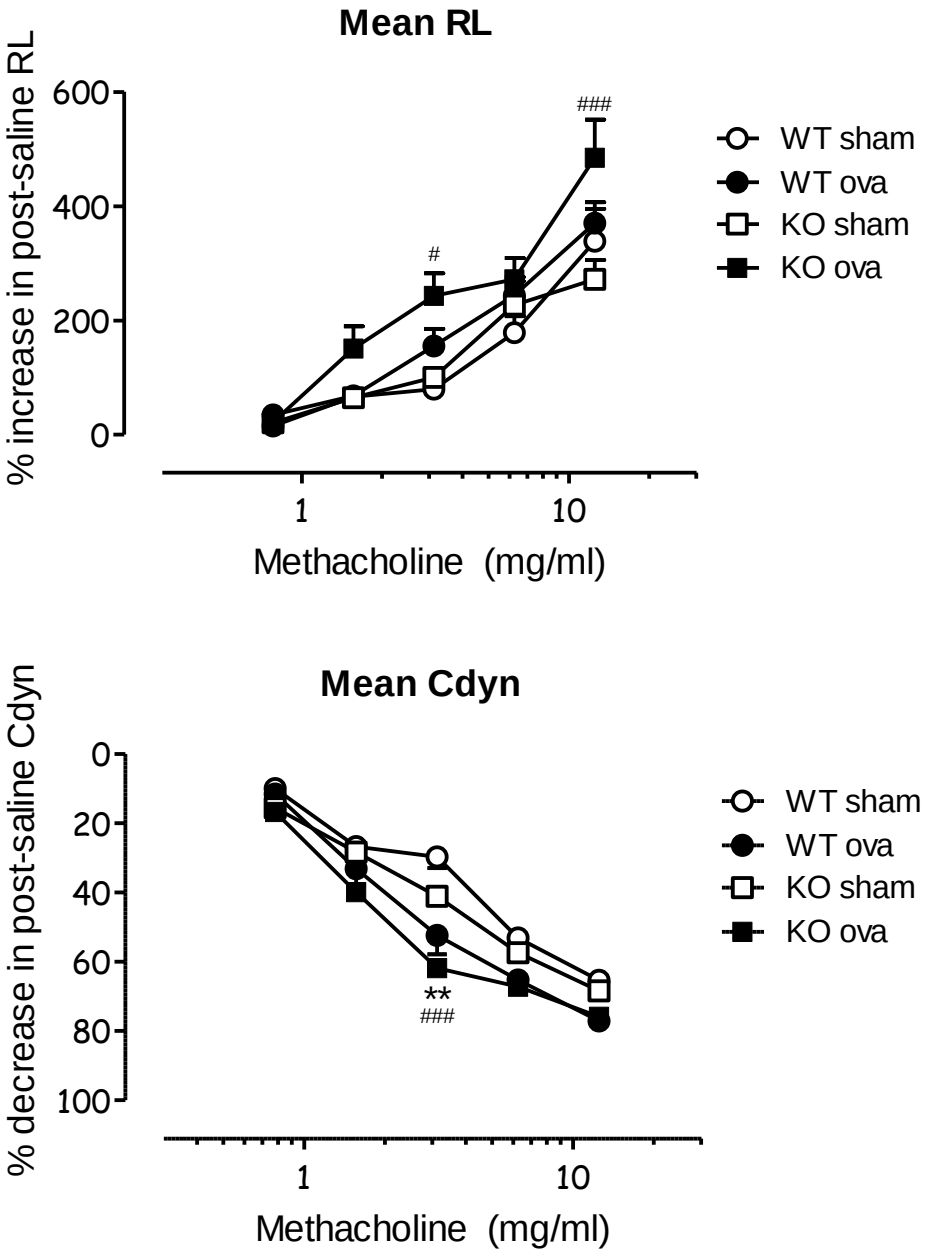
4.3.6-1 Sham mice were immunised with 0.4ml saline and aluminium hydroxide once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge levels of cytokines were assessed in the BAL fluid. Data represents mean ± SEM of n=15-16. No pattern bars represent wild type mice, chequered bars represent SERCA2$^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. A two way ANOVA was performed with a Sidak post hoc analysis; *p<0.05, **p<0.01, ***p<0.001.

4.3.7 Effect of ovalbumin immunisation on lung function parameters

Wild type and SERCA2$^{+/-}$ mice both exhibited concentration dependent increases in total lung resistance following methacholine administration (Fig. 4.3.7-1, upper graph). Immunisation with ovalbumin significantly enhanced the percentage increase in total lung resistance above basal levels at 3.12 mg/ml and 12.5 mg/ml methacholine, (p=0.05 and p<0.001 respectively) in the SERCA2$^{+/-}$ mice but not the wild type mice.

Dynamic compliance (Cdyn) was dose dependently decreased by methacholine in all four groups (Fig. 4.3.7-1, lower graph). Immunisation with ovalbumin significantly reduced Cdyn in the wild type mice compared with sham immunised mice at 3.12 mg/ml methacholine (p<0.01) again confirming the presence

of BHR in wild type immunised mice following antigen challenge. The effect of immunisation was only subtly enhanced in the knockout mice with a significant reduction at 3.12 mg/ml methacholine ($p<0.001$).

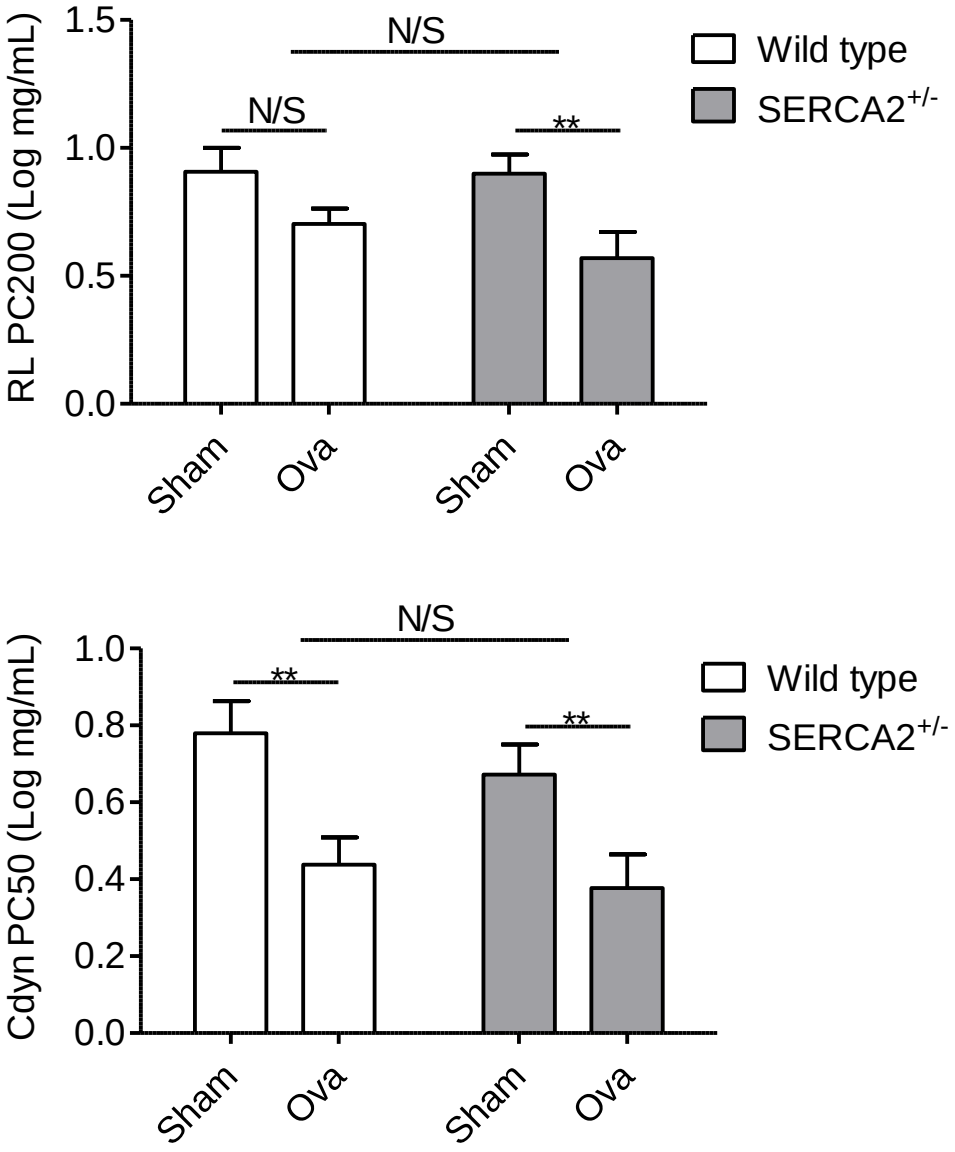


4.3.7-1 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge airway resistance (RL, upper graph) and dynamic compliance (Cdyn, lower graph) were measured. Data represents mean ± SEM of n=19-24 per group. Data analysed by repeated measures two-way ANOVA; **p<0.01 WT/sham vs WT/OVA and #p<0.05, ###p<0.001 KO/sham vs KO/OVA.

The RL PC200 is the concentration of methacholine required to increase airway resistance 200% above the saline induced baseline level. A two way ANOVA with genotype and immunisation as the fixed effects showed that there was a significant difference in RL PC200 following immunisation ($p=0.003$) but

not between genotypes or an interaction between the two (p=0.42 and p=0.47 respectively). Following sensitisation with ovalbumin there was an increase in sensitivity to methacholine in both the wild type and knockout mice. The SERCA2$^{+/-}$ mice, following immunisation, exhibited a significantly enhanced sensitivity to methacholine increasing their airway resistance whereas the wild type mice did not (p=0.006 and p=0.11 by Sidak post-test respectively, Fig. 4.3.7-2, upper).

Dynamic compliance was similarly affected by immunisation to ovalbumin as analysed by two way ANOVA, showing an increased sensitivity demonstrated by a lower concentration of methacholine required to decrease compliance by 50% from baseline (Cdyn PC50, p=0.0002, Fig. 4.3.7-2, lower). Genotype however did not affect the result (p=0.30) and there was no interaction with immunisation status (p=0.74). Contrary to airway resistance however there appeared to be no difference between the changes in sensitivity between the wild type mice and the SERCA2$^{+/-}$ mice following immunisation.



4.3.7-2 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge airway resistance was derived from analysing changes in flow using a pneumotachograph. Data represents mean ± SEM of n=19-24 per group. A two way ANOVA was performed with a Sidak post hoc analysis **p<0.01.

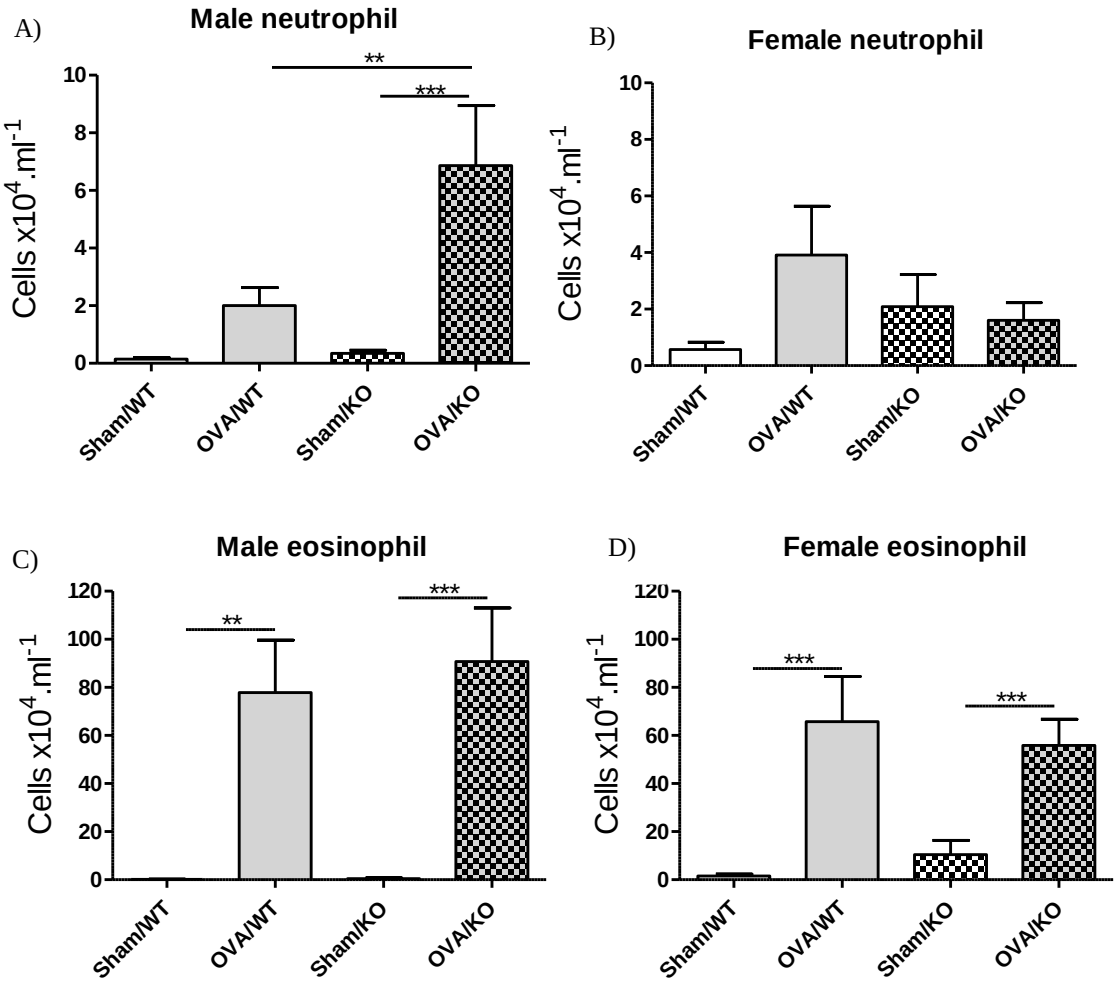
4.3.8 The impact of gender and immunisation on heterozygous SERCA2$^{+/-}$ mice

It has been shown previously that gender can affect the asthmatic phenotype (Choi, 2011; Melgert *et al.*, 2005). Therefore the data was analysed for gender differences for the different variables measured.

4.3.8.1 Impact of gender on neutrophil infiltration into the airways

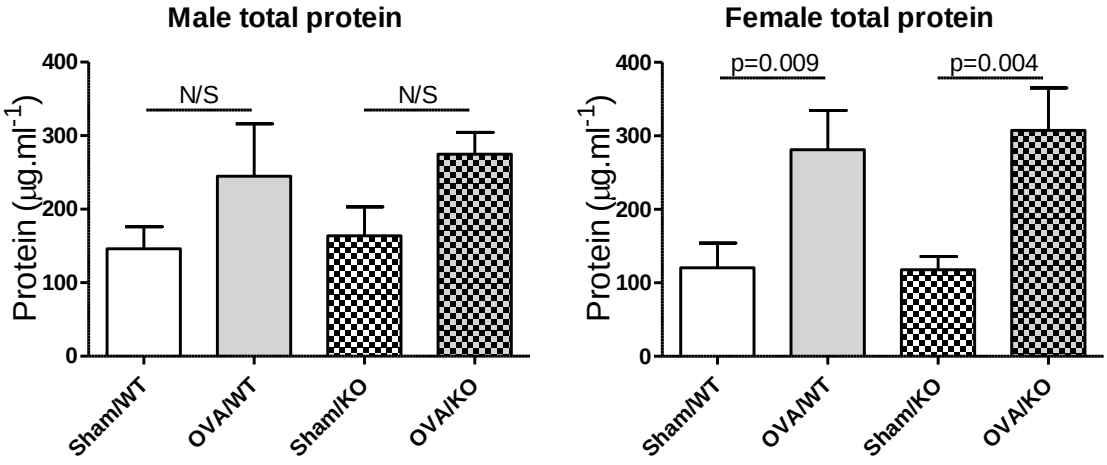
An interesting dichotomy appeared when reanalysing the cellular recruitment to the lungs after analysing for gender and analysing by three way ANOVA with a Sidak post-test. Immunisation significantly affected the outcome ($p<0.001$) with a further three way interaction between each factor ($p=0.006$). As

shown with the combined data in Fig. 4.3.3-1 a reduction of SERCA2 in the knockout mice led to a trend of enhanced neutrophilia in the sham immunised mice. Following ovalbumin immunisation, there was no significant change between the wild type and knockout female mice (p=0.12). On the other hand there was significantly more neutrophils in the BAL of male knockout ovalbumin immunised mice compared to their wild type controls (6.86 ± 2.09 neutrophils $x10^4.ml^{-1}$ and 2.0 ± 0.63 neutrophils $x10^4.ml^{-1}$ respectively, p=0.004, Fig. 4.3.8-1 below). The enhanced neutrophilia in the knockout ovalbumin immunised mice is also shown by significant increase compared to the sham knockouts (p<0.001), the immunisation effect was not significant in the wild types (p=0.259). Immunisation status significantly affected eosinophil recruitment (p<0.001) while contrary to the neutrophil data gender and genotype did not have an impact (p=0.34 and p=0.87 respectively).



4.3.8-1 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge neutrophil (A+B) and eosinophil (C+D) levels were assessed in the BAL fluid (A+C) males and (B+D) females. Data represents mean ± SEM of n=9-15 per group. No pattern bars represent wild type mice, chequered bars represent $SERCA2^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. **p<0.01, ***p<0.001. A three way ANOVA was performed with a Sidak post hoc analysis.

Analysis of the total protein concentration within the airways by three way ANOVA revealed a significant immunisation effect ($p<0.001$) but no significant gender or genotype differences ($p=0.98$ and $p=0.55$ respectively, Fig. 4.3.8-2). The only disparity between the genders was the more variable increase in total protein in the male mice compared to the females following ovalbumin immunisation compared to sham. Neither wild type nor knockout male OVA immunised mice had significantly greater total protein levels in the BAL fluid compared to their sham controls however the female mice did ($p=0.009$ and $p=0.004$ respectively). Four of the samples from the male groups were contaminated with blood leading to very high protein levels being recorded which were omitted from analysis. The repeats in this group therefore dropped from eight to six resulting in less power (0.987 females, 0.567 males) in the statistical test and a higher chance of a false-negative outcome i.e. a type two error.



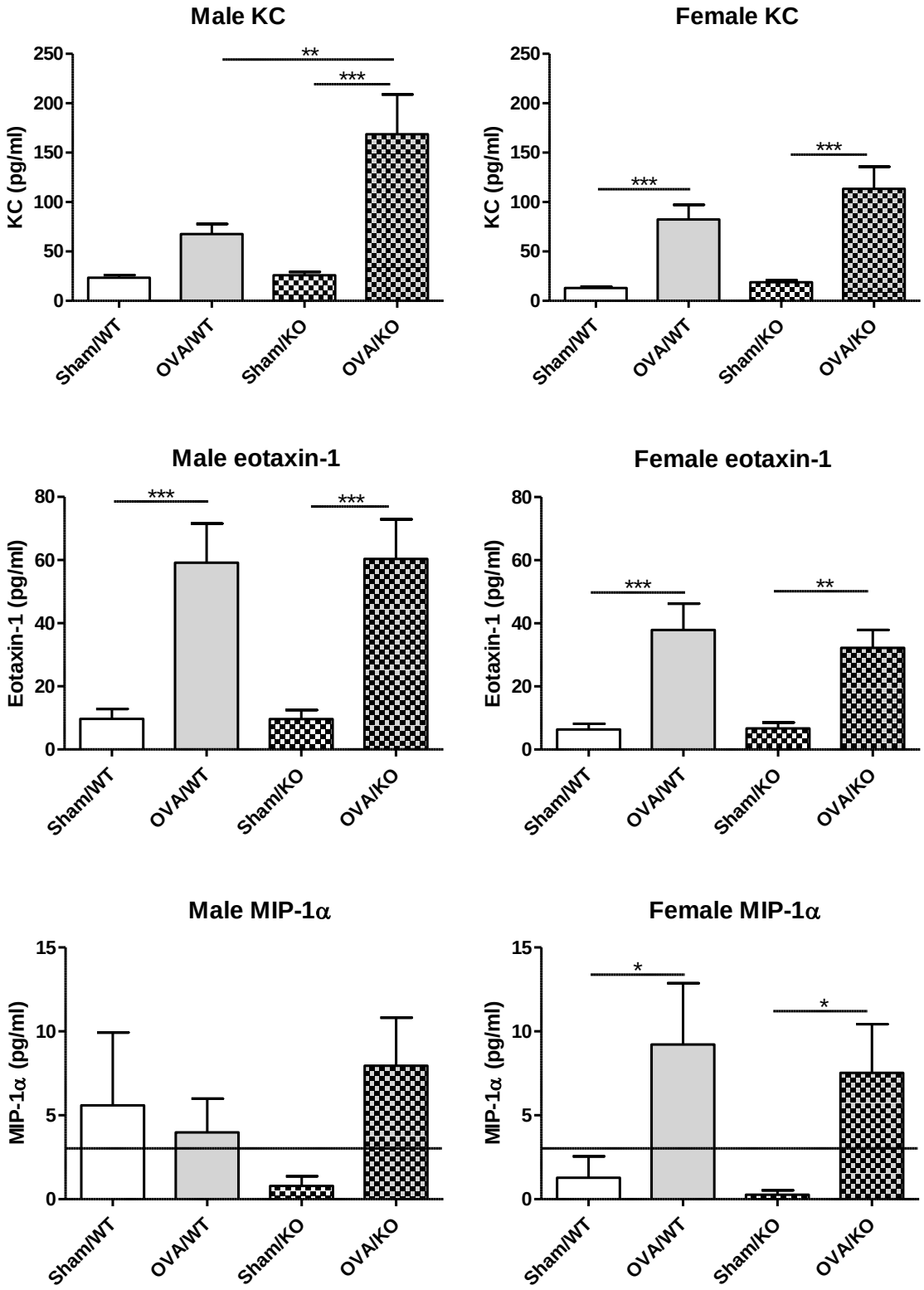
4.3.8-2 Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge total protein levels were assessed in the BAL fluid. Data represents mean ± SEM of n=6-8 per group in duplicates. No pattern bars represent wild type mice, chequered bars represent SERCA2$^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. Data analysed as a three way ANOVA with genotype and immunisation as the fixed effects and a Sidak post-hoc analysis.

4.3.8.2 Impact of gender on cytokine recruitment to the airways

KC is one of the major chemokines for neutrophil recruitment into the lungs and their presence in the male mice airways reflected this. Akin to the neutrophils there was a significant interaction between immunisation status and genotype ($p=0.018$, Fig. 4.3.8-3). A post-hoc test showed a significant immunisation effect only in the male knockout mice ($p<0.001$) and not the wild type mice ($p=0.20$).

Further following the pattern of the male neutrophilia data a significant difference existed between the wild type OVA sensitised mice and the knockout OVA sensitised mice ($p=0.003$).

Regarding eotaxin-1 presence in the airways there was a significant immunisation and gender effect ($p<0.001$ and $p=0.01$ respectively) but no genotype effect. The male ovalbumin immunised mice displayed enhanced eotaxin-1 levels within the BAL fluid compared to the females. Several sham immunised groups fell below the detection limit for MIP-1α expression therefore comparisons should be made with caution. That said, the female mice exhibited an immunisation effect ($p=0.004$), whereas the male mice did not.

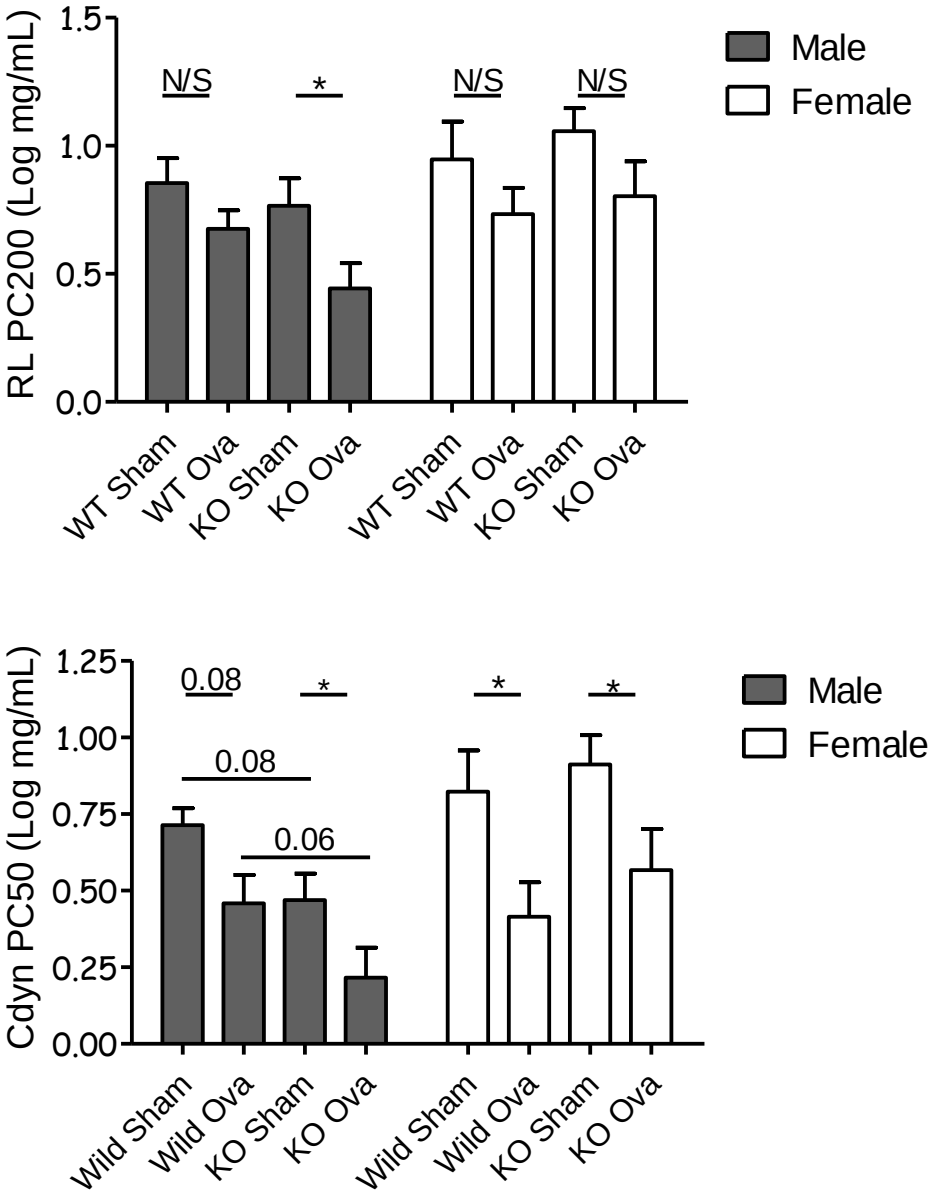


4.3.8-3 Sham mice were immunised with 0.4ml saline and aluminium hydroxide once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge levels of cytokines were assessed in the BAL fluid. Data represents mean ± SEM of n=7-8. No pattern bars represent wild type mice, chequered bars represent SERCA2$^{+/-}$ mice, white bars were immunised with the sham control and grey bars with ovalbumin. A three way ANOVA was performed with a Sidak post hoc analysis.

4.3.8.3 Impact of gender on airway resistance

The larger impact of genotype on the male mice present in the neutrophil recruitment to the BAL fluid was also present in lung function parameters measured (Fig. 4.3.8-4). The male knockout mice were the most affected in terms of airways sensitivity to methacholine as measured by Cdyn PC50 and RL PC200. A three way ANOVA was performed with gender, genotype and immunisation status as the fixed effects and a blocking factor to control for variability between different experimental days. A significant gender ($p=0.003$) and immunisation effect ($p=0.005$) was observed. Sidak post-hoc analysis revealed a significant difference between male knockout sham and OVA immunised groups ($p=0.023$).

A three way ANOVA performed on the Cdyn Log PC50 data showed a significant gender ($p=0.002$), immunisation ($p=0.0001$) and some gender genotype interaction ($p=0.061$). A Sidak post-hoc analysis revealed significant differences in the female wild type and knockout, sham versus ovalbumin ($p=0.001$ and $p=0.01$ respectively). There were no such differences observed in the male mice between immunisation status in either the wild type ($p=0.193$) or knockout ($p=0.086$). The sham treated male knockout mice had a lower Cdyn PC50 compared to their wild type counter parts suggesting an inherent sensitivity which was further enhanced after ovalbumin immunisation.

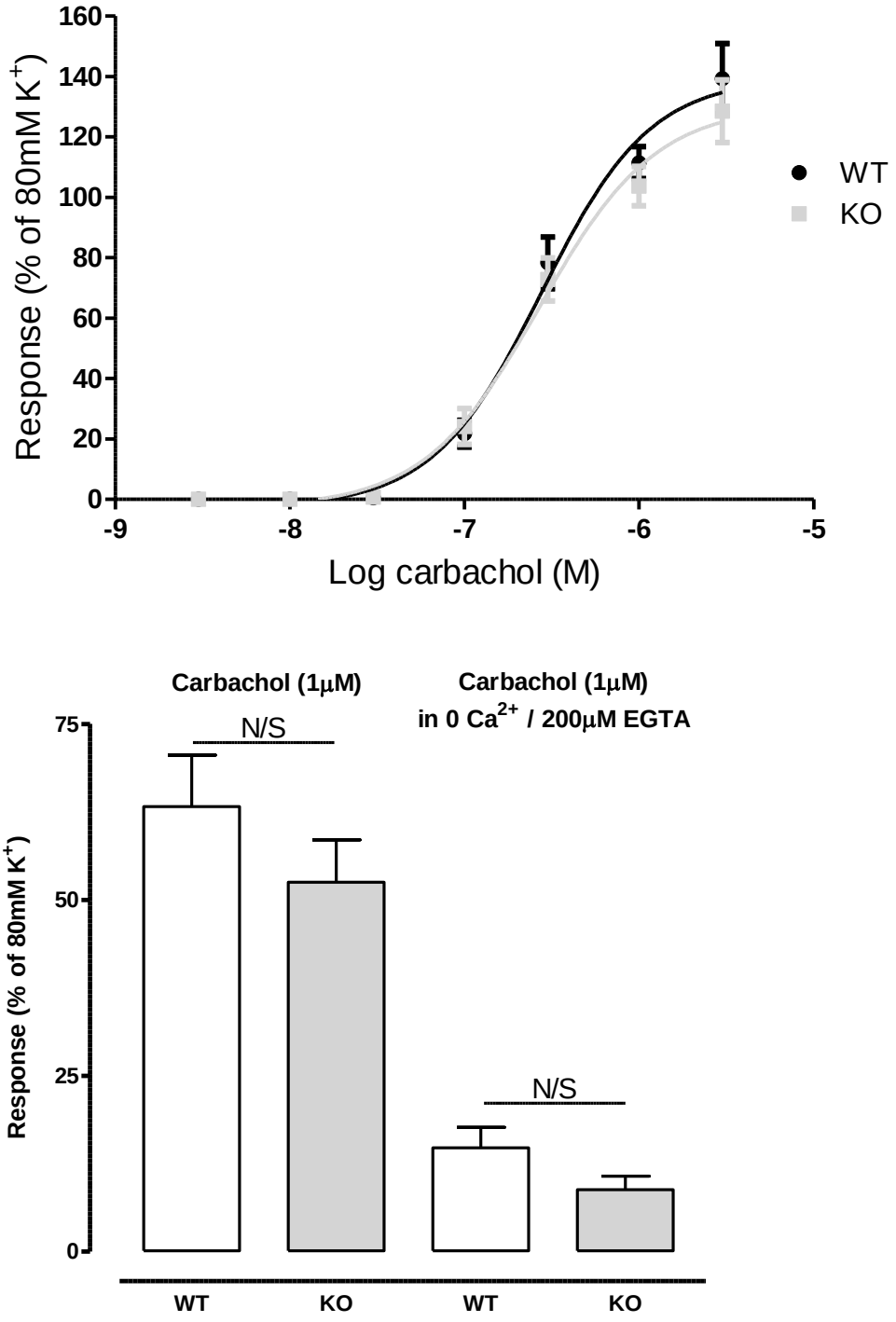


4.3.8-4: Mice were immunised with 30µg ovalbumin once a week for four weeks then challenged to 3% ovalbumin twice a day for four days. Twenty-four hours post final challenge log RLPC200 (upper) and log Cdyn PC50 (lower) were derived from analysing changes in flow using a pneumotachograph. Data represents mean ± SEM of n=8-13 per group. *p<0.05, **p<0.01, ***p<0.001. Data was analysed by three way ANOVA with gender, genotype and immunisation as the fixed effects and a Sidak post-hoc analysis.

4.3.9 Myography

Airway sensitivity and reactivity between the wild type and knockout mice were measured *ex vivo* by wire myography in response to the muscarinic acetylcholine receptor agonist carbachol (Fig. 4.3.9-1). Intrapulmonary sections of primary bronchioles were dissected from wild type and SERCA2$^{+/-}$ naive mice. The sections were then mounted on a myograph and challenged with 80mM K^+ three times. Rising carbachol concentrations were added to make a response curve with peak tensions normalised to the final

80mM K^+ response. Data represents mean ± SEM, n=5. There was no change in EC_{50} between wild type and knockout mice 276 nM (95% CI: 200-381) and 270 nM (95% CI: 190-384) respectively (p=0.97 analysing logEC50 by t-test). There was also no change in the maximum response between the wild types (139 ± 8.7 and 130 ± 8.8 % of high K^+ respectively, p=0.74 by t-test). The 1μM carbachol dose was repeated in calcium ion free extracellular solution to isolate the store release component of calcium mobilisation. Genotype did not affect the subsequent contraction.



4.3.9-1 Intrapulmonary sections of primary bronchioles were dissected from wild type and SERCA2$^{+/-}$ naive mice. The sections were then mounted on a myograph and challenged with 80mM K^+ three times. Rising carbachol concentrations were added to make a response curve with peak tensions normalised to the final 80mM K^+ response. Data represents mean ± SEM, n=5, grey bars represent knockout mice. Student's t-test was performed to analyse difference between means following 1μM carbachol in the presence (left) or absence (right) of Ca^{2+} in the Kreb's solution (lower graph).

4.4 Discussion

The results outlined in this chapter reveal a subtle role for SERCA2 in this *in vivo* model of asthma. There is an increased neutrophilia in the naive knockout mice compared to the wild types which is greatly enhanced only in the males following sensitisation and challenge to ovalbumin. The presence of KC in the airways is reflective of this and is also elevated in the male mice with reduced SERCA2 expression. A concomitant subtle increase in sensitivity to methacholine was observed in allergic mice that was again greatest in male SERCA2 knockout mice.

Initially it was confirmed the knockout mice had a reduced protein expression of SERCA2 in the homogenised lungs as had been shown previously in the heart (Periasamy *et al.*, 1999). Interestingly it appeared as if immunisation with ovalbumin also increased SERCA2 expression however this was not significant.

4.4.1 Inflammatory cell infiltrate

Immunisation with ovalbumin in the wild type mice resulted in a robust, significant increase in the total cell infiltrate and each of the three leukocytes measured within the BAL. Although this allergic model has been very well established previously (DiGiovanni *et al.*, 2009; Fernandez-Rodriguez *et al.*, 2010; Reddy *et al.*, 2012) no work had been performed on the Black Swiss 129/SVJ mixed strain. Therefore a window had to be established to investigate the potential effect of genotype which was achieved in the corresponding preliminary work (Ojo, 2011).

No genotype immunisation status interaction was observed in the recruitment of eosinophils into the airways contrary to the hypothesis derived from the *in vitro* data involving the chemotactic agent eotaxin-1 (Mahn *et al.*, 2009). In that publication a reduction of SERCA2 expression lead to an increase in eotaxin-1 following IL-13 stimulation. Following ovalbumin challenge in both genotype groups there was a two fold increase in total protein in the airways of which approximately 70% of the leukocytes were eosinophils. Such a large, possibly saturating response may have masked any genotype differences in both the eosinophilia and total protein end-points. A milder immunisation and challenge protocol was tested in the preliminary run but no significant change in leukocyte recruitment or airway function was observed. Further characterisation work is required to optimise the response in this mouse strain. It is possible that the less than 50% reduction in SERCA2 protein expression observed in these mice was not sufficient to influence cell recruitment and cytokine release in the pooled data of this model of allergic inflammation.

Neutrophil recruitment was elevated following immunisation in both the wild type and knockout mice compared to the sham groups. A subtle elevation was also apparent in the knockout mice compared to the wild types however the genotype did not affect the result ($p=0.14$), nor was there an interaction with the immunisation status. In a subset of severe asthmatic patients the airway remodelling observed has been linked with a neutrophilic inflammation (Gupta *et al.*, 2010; Wenzel *et al.*, 1999). The increase in neutrophilia recovered from the airways in the BAL is reflected by elevated levels of polymorphonuclear leukocytes in the surrounding lung parenchyma demonstrated by the H&E staining. The lack of observed genotype impact on the BAL fluid cell data could be because of the sampling technique used. Cells were stained using haematoxylin and eosin and differentiated by eye-sight. Although this method is reproducible with a trained eye a further quantification using a neutrophil specific stain such as neutrophil elastase, followed by blind quantification would provide a more robust analysis.

It is interesting that there appears to be a subtle increase in neutrophilia in the sham immunised knockout mice, thus suggesting that it is purely a result of the reduced SERCA2 expression. As the knockouts are global and every cell type will have a reduced expression it is impossible to know what specifically is driving the infiltration. If the inflammation present in the parenchyma is neutrophilic it is possible that the ASM cells are contributing more KC (John *et al.*, 1998; Pang *et al.*, 1998), a neutrophil chemotactic as a result of reduced SERCA2. Equally the reduction in SERCA2 could lead to the neutrophils themselves have a higher propensity for migration or enhanced KC release from the epithelium or inflammatory cells. It has been shown that in mice neutrophil chemotaxis mediated by CXCR2 (KC is a ligand for) is in part dependent upon TRPC6 expression (Lindemann *et al.*, 2013). It is plausible TRPC6 is increased in the SERCA2 knockout mice or the enhanced chemotaxis is a more general result of altered calcium handling affecting downstream mediators such as AKT or MAPK. Using a conditional SERCA2 (flox) allele to specifically knockdown expression in ASM or elsewhere would help to answer this question (Andersson *et al.*, 2009). The inducible nature of the knockdown would also circumnavigate the problem of other calcium handling proteins such as TRP channels and the sodium/calcium exchanger compensating for reduced SERCA2 expression from birth, thus masking potential effects. Reducing SERCA2 *in vitro* using siRNA and stimulating with a KC inducing agent such as IL-1β in ASM could also shed light on the neutrophilia observed.

4.4.2 Chronic ovalbumin challenge

Chronic models have been developed to look at airway wall remodelling which can manifest itself early in a patient's life (Barbato *et al.*, 2006) and plays an important role in the pathophysiology of the disease. It

is classified as an increase in airway wall thickness, comprised of collagen deposition, goblet cell hyperplasia, myofibroblast hyperplasia and epithelial hypertrophy (Busse *et al.*, 1999; Knight, 2001). Both ovalbumin (McMillan *et al.*, 2004) and house dust mite (Johnson *et al.*, 2004a) can be used to investigate this aspect of the disease.

As hypothesised, using a model of acute asthma resulted in no treatment effect on airway remodelling as observed by collagen and ASM deposition. A chronic model to induce airway remodelling would have been very interesting to use with these mice as it resembles the clinical features of asthma more readily as well as providing a more accurate tool for testing drug effectiveness (Evans *et al.*, 2012). Unfortunately due to problems in the animal house severe delays were incurred on the *in vivo* aspect of the investigation, resulting in time for only the acute asthma model to be used.

The enhanced ASM proliferation observed *in vitro* when SERCA2 protein expression is diminished using siRNA (Mahn *et al.*, 2009) provides rationale that remodelling would be enhanced in this model after chronic allergen exposure. I hypothesised that in the sham $SERCA2^{+/-}$ mice there would be pulmonary morphological changes compared to the wild type mice as a result of reduced SERCA2 expression from birth. There appeared to be no difference in the smooth muscle layer between the two groups disproving the hypothesis. Perhaps due to compensatory mechanisms as mentioned previously ensuring that the dynamics of calcium handling remains reasonably unaltered in spite of the reduced expression. Culturing the smooth muscle cells from the knockout mice and measuring calcium transients with fura would show to what extent the homeostasis is altered.

4.4.3 Cytokine data

Interestingly no IL-17, TNF-α or IL-13 was detectable in the BAL fluid in any of the groups contrary to the rise often seen following ovalbumin sensitisation and challenge (Asano *et al.*, 2010; Li *et al.*, 2012; Melgert *et al.*, 2005; Quan *et al.*, 2012; Xu *et al.*, 1997). None of these previous publications used the 129SVJ/Black Swiss mice used in the present study and therefore the lack of response may be due to genetic variation within the strain. It could also be due to a suboptimal time point being used to sample the inflammatory cytokines. The articles previously stated all sacrificed the mice 24 hours after the final challenge, similar to as performed here so it is unlikely to be the case but this would need to be investigated in this particular strain. High IL-17A concentrations have been observed in severe asthmatics with high IL-8 and neutrophilia and it has been suggested that T_H17 cells contribute to this (Bullens *et al.*, 2006). As the current model supports a severe asthmatic phenotype with enhanced neutrophilia it is

therefore surprising IL-17 levels were not detectable within the BAL fluid however this may again be a strain dependent effect. C3H mice produce very little IL-17A in response to allergen challenge whereas A/J mice are much more susceptible (Lajoie *et al.*, 2010).

The levels of the chemokines, eotaxin-1 and KC present in the murine airways were consistent with the pulmonary recruitment of eosinophils and neutrophils respectively. As with the eosinophils there was a possibly saturating recruitment of eotaxin-1 observed following ovalbumin sensitisation and challenge. Any putative "left-ward shift" in the dose response curve would be unobserved as maximal levels of recruitment are being achieved in both genotypes. ASM cells derived from healthy patients which had SERCA2 protein expression reduced to similar levels as observed in these knockout mice exhibited enhanced eotaxin-1 production upon IL-13 stimulation (Mahn *et al.*, 2009). The fact that no additional eotaxin-1 was present in the naive $SERCA2^{+/-}$ mice compared to the wild type could be explained by the lack of detectable IL-13 in the airways.

Unlike the neutrophil data analysed from the BAL, a genotype effect and interaction with immunisation was picked up in the KC protein levels measured by luminex bead assay ($p=0.02$). The technique is a more powerful analysis and the finding further backs up the previous point raised that a type 2 error may have occurred and that a genotype immunisation interaction does exist in the neutrophil recruitment. The enhanced KC production in the OVA/KO group compared to the OVA/wild type group ($p<0.001$) is a very interesting finding and indicates a role for calcium homeostasis controlling the secretion of KC into the airways which may be altering neutrophil recruitment into the lungs.

Macrophage inflammatory protein-1α (MIP-1α/CCL3) was barely detectable in the BAL fluid but did appear to be elevated following immunisation however due to most of the data being below the detection limits of the assay steadfast conclusions cannot be drawn. It has been shown before that it is increased following ovalbumin sensitisation and challenge however the levels obtained in this study were markedly lower (Li *et al.*, 2004). It can be produced by most mature hematopoietic cells such as neutrophils, dendritic cells, mast cells and T and B cells and act on its CCR1 receptor to aid the chemotaxis of monocytes, T-cells, neutrophils and eosinophils (Maurer *et al.*, 2004). MIP-1α production usually requires activation from cytokines such as TNF-α so the low levels may be a result of reduced TNF-α present as none was detectable in the BAL, possibly as a strain dependent genetic effect of the mice used.

4.4.4 Lung function

Ovalbumin immunised and challenged mice demonstrated a trend towards increased airways responsiveness to methacholine as determined by changes in total lung resistance and dynamic compliance, however significance was only achieved in a few parameters tested. Airway responsiveness to methacholine (as expressed as % change in total lung resistance) was significantly elevated following ovalbumin immunisation in the SERCA2$^{+/-}$ mice but not in the wild types. Reflective of this the knockout mice displayed an increased sensitivity to methacholine shown by the significant decrease in RL PC200 ($p<0.05$ Fig. 4.3.8-4). It is difficult to determine whether airway hyper-responsiveness occurs through inherent changes in the ASM manifested as enhanced sensitivity to spasmogens or via alterations in the pulmonary environment (e.g. mucus secretion, oedema, loss in airway surface tension, inflammation). A study using tracheal preparations from ovalbumin sensitised mice and a variety of agonists suggested that AHR involves specific changes in the muscarinic pathway in ASM, possibly at the coupling to contractile machinery (Fernandez-Rodriguez *et al.*, 2010). Only methacholine, a muscarinic acetylcholine receptor agonist was used, so the AHR observed after ovalbumin sensitisation fits with this work. As knockdown of SERCA2 would result in changes downstream to the receptor the observed sensitivity should remain after stimulation to an array of contractile agonists.

A greater genotype difference in methacholine responsiveness as measured by changes in total lung resistance was observed compared with changes in dynamic compliance suggesting that the effect was primarily in the proximal airways (Irvin *et al.*, 2003). It is thought that this is a result of airway narrowing whereas Cdyn is more influenced by the elastic recoil of the lungs (Takeda *et al.*, 2001). One might expect that using the chronic model would result in a larger decrease in dynamic compliance as more remodelling occurs which could primarily affect the smaller airways.

4.4.5 Effect of gender

Another important genetic variation in asthma is the effect gender plays on its pathogenesis. It is well described that boys have a higher incidence of asthma than girls however around adolescence and puberty the pattern is reversed and females have the higher incidence (Choi, 2011). It has been hypothesised that this occurs because of slower pulmonary development in males, sex hormones, as those from males have been shown to suppress asthma whereas female sex hormones aggravate it and perhaps gender specific environmental exposures (Almqvist *et al.*, 2008; Osman, 2003). The mice used in this study were on average 90 days old at the time of experimentation. Male mice typically reach puberty at seven weeks and

females at six weeks therefore the mice used in this study were into their adult life. The murine oestrous cycle lasts between 4-5 days therefore litter matching between groups was important to reduce variation.

Contrary to the evidence in human asthma it was the male knockout mice in this particular strain which demonstrated the more overt asthmatic phenotype. In the ovalbumin sensitised male mice the knockouts had a significantly greater KC and neutrophil infiltration into the airways and a much higher sensitivity to methacholine increasing airway resistance and decreasing dynamic compliance. Genotype and gender on the other hand had no impact on eosinophil recruitment however there was a gender-immunisation interaction regarding eotaxin-1 but no association with the genotype. Thus suggesting the enhanced effect of reduced SERCA2 expression was a neutrophil specific phenomenon. Additionally the naive male knockout mice had a significantly lower Cdyn PC50. The data partly fits in with previous data published using a similar protocol investigating sex differences in BALB/c mice (Melgert *et al.*, 2005). The paper concludes that female mice are more susceptible to inflammation however the male mice are more sensitive to methacholine measured by a decreased PC300 Penh value. In a study using gonadectomised male rats a decrease in Ca^{2+} transient amplitude and a prolonged decay rate was observed in cardiomyocytes which was restored with testosterone replacement therapy (Curl *et al.*, 2009). It was suggested that this could be due to a decrease in SERCA2 expression or function in which case you might expect female SERCA2 knockout mice to have even less SERCA2 function manifested as an enhanced phenotype in this model which wasn't the case. It has been shown that the amount of α-SMA is similar in male and female mice and there is no interaction between gender and immunisation ruling out the possibility of the knockout exerting a greater effect in the males due to a larger ASM volume (Blacquiere *et al.*, 2010).

4.4.6 Ex vivo contractile response

A concentration response curve was performed using myography on naive mice to directly assess the contractility of primary bronchioles from wild type and $SERCA2^{+/-}$ mice. Male mice were used as the greatest *in vivo* differences were observed with them however no change in EC_{50} or maximum response occurred. A sub-maximal dose of carbachol was used in the absence of calcium to negate any calcium entry and just assess store content which could be lowered in the knockout mice but again there was no difference. Although SERCA2 protein expression has been reduced in the knockout mice, from the data in this chapter it is impossible to know how much the calcium handling dynamics have been altered. Furthermore, a reduced SERCA2 expression and subsequent calcium ion reuptake into the sarcoplasmic reticulum by no means guarantees enhanced contraction. The sarcoplasmic reticulum forms a superficial

barrier which divides the cytosol into functionally distinct spaces (Janssen *et al.*, 1999). It is changes in Ca^{2+} in the deep cytosol and not subsarcolemmal spaces which are more correlated with contraction. Using CPA, a SERCA2 antagonist elevated Ca^{2+} in the deep cytosol resulting in contraction but no change in membrane currents (Janssen *et al.*, 1999). The SERCA2 knockout mice would be expected to behave in a similar fashion. However without knowing the full protein expression changes which may compensate for this loss the actual alterations in calcium handling could be more pronounced near the plasma membrane.

4.4.7 Caveats

A major drawback to this study and every one like it are that significant differences exist between murine and hominine immune system development and activation (Mestas *et al.*, 2004). Additionally the pulmonary physiology of mice are vastly different to that in humans primarily due to their differing anatomy (Metzger *et al.*, 2008). Therefore any conclusions made cannot be simply assumed to occur in humans however with the complementary *in vitro* data collected from primary cultured human ASM cells an overall picture is starting to be built. Furthermore this model is only looking at the contribution of SERCA2 in an allergen-induced model of asthma, chosen because of the strong associations with specific IgE reactions and hypersensitivity to aeroallergens (Burrows *et al.*, 1989). There are arguments however, that atopy is a secondary consequence to asthma, therefore the current model lacks gaining insight into the role of SERCA2 into other potential "drivers of susceptibility" (Burrows *et al.*, 1995; Martinez *et al.*, 2013).

A second caveat is that the knockdown of SERCA2 was across the whole body and from birth. The *in vitro* data collected focuses upon the impact of reduced SERCA2 expression in ASM only and the impact that has on calcium homeostasis and the development of an asthmatic phenotype. The data presented in this chapter backs up the hypothesis that a reduction of SERCA2 can enhance many phenotypes associated with asthma however it can't determine how responsible the ASM is for this and to what degree calcium homeostasis is perturbed in it. The fact that the reduction occurs throughout the organism's life span allows other calcium handling proteins to potentially change their activity to mitigate the loss of function. Work has not been done to see whether SERCA2 reduction precedes the development of asthma but this would be an invaluable addition to the field.

The strain used is also unfortunate as it isn't particularly responsive to this model compared to C57/BL6 and BALB/c mice. As discussed before a more pressing drawback is the discrepancy between all murine models and the human clinical setting. So the work here, although might not be as striking as would be

seen in C57/BL6 mice, provides evidence that SERCA2 disruption can enhance the asthmatic phenotype *in vivo*.

4.4.8 Future studies

- Culturing the ASM cells derived from the knockout mice and measuring changes in intracellular calcium concentration in response to several agonists to determine the extent calcium handling is altered.
- A chronic ovalbumin challenge protocol to understand the role calcium homeostasis plays in airway remodelling *in vivo*.

4.4.9 Conclusion

In conclusion this chapter demonstrates that reduced SERCA2 expression enhances neutrophilia, airway resistance and decreases dynamic compliance primarily in male mice. The results are largely in line with the hypothesis however none of the changes were as dramatic as expected. Using a chronic model to drive airway remodelling is likely to show a greater impact of disrupted calcium handling as the previous *in vitro* data suggests enhanced proliferation and cell spreading (Mahn *et al.*, 2009).

Chapter 5 The effect of β-adrenergic agonists on ASM calcium homeostasis

5.1 Introduction

5.1.1 β_2-adrenergic receptor agonists in asthma

β_2-adrenergic receptor (β_2-AR) agonists form a vital part of treatment for the majority of asthmatics, as they offer a quick onset of action in relaxing ASM and have a good safety profile. These agonists shift the equilibrium of the β_2-AR in favour of its activated form from its resting inactive state thereby increasing the number of receptors associated with the G_s protein and GTP (Johnson, 2001). Receptor activation results in numerous downstream signalling events, most importantly an adenylyl cyclase mediated increase in cAMP, followed by activation of PKA and muscle relaxation.

As the first generation of these drugs, including salbutamol, have a maximum duration of action of only four hours, long acting β_2-AR agonists (LABAs) were developed particularly for patients whom attacks were worst at night or morning. However, the safety of formoterol and other LABAs was called into question following accumulating evidence of an increased risk of morbidity and mortality (Nelson *et al.*, 2006; Rodrigo *et al.*, 2009; Salpeter *et al.*, 2006; Spitzer *et al.*, 1992). The level of concern has resulted in an (albeit controversial) FDA mandated trial on its safety (Sears, 2013). In a similar fashion, β- agonists, which improve cardiac contractility in patients with congestive heart failure (CHF) in the short term were found to cause increased mortality with chronic use, whereas β-blockers went from being contraindicated in CHF to being approved as the drug of choice in treatment (Sorrentino, 2003; Waagstein *et al.*, 1975). Thus, parallels have recently been drawn between CHF and asthma, suggesting that "biased agonism" may lead to agonists acting on the conventional G-protein secondary messengers to evoke cyclic AMP-dependent effects but also on the β-arrestin-2 pathway creating secondary effects (Walker *et al.*, 2011).

5.1.2 The potential of β_2-AR to alter calcium homeostasis

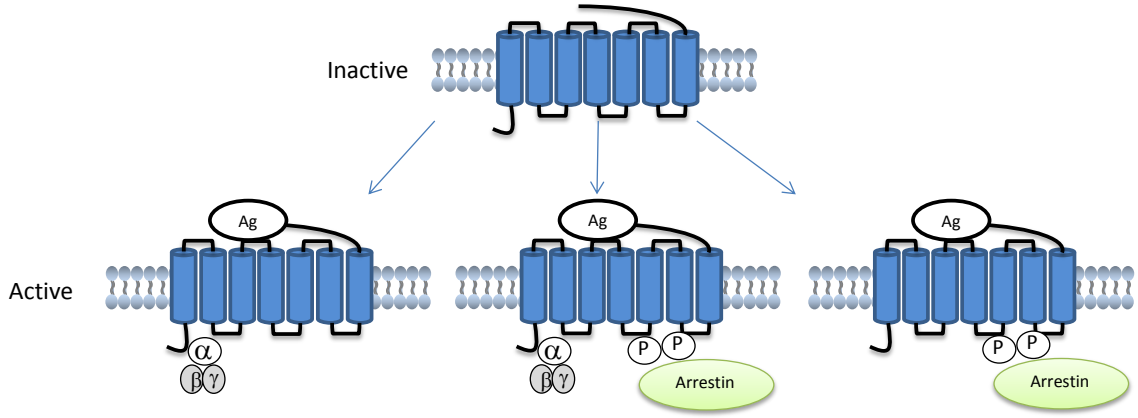
Interestingly it has recently been shown that prolonged exposure of formoterol can lead to a 50% decrease in SERCA2a protein in the heart (Ryall *et al.*, 2008). It is thought that this reduction in SERCA2a is due to a decline in gene transcription, possibly via glycogen synthase kinase-3β (GSK-3β) which has been implicated as a negative regulator of SERCA2 gene transcription (Michael *et al.*, 2004). Ryall *et al.* showed a 40% decrease in its phosphorylation upon β_2-AR stimulation, an effect which enables more of the active form to directly interact with SERCA's promoter region and inhibit its transcription. It is possible that this secondary effect is mediated via a secondary intracellular pathway such as β-arrestin-2.

It has previously been shown that SERCA2 expression is reduced in ASM from asthmatic patients and that this contributes to the asthmatic phenotype (Mahn et al., 2009). It would therefore be predicted from the above that treatment of such patients with a LABA might further depress SERCA2 expression and worsen the condition; this would have clear implications for therapy. Indeed, Ojo reported in his PhD thesis (Ojo, 2011) that both formoterol and salbutamol decreased expression of SERCA2 in healthy cultured ASM cells in a concentration dependent fashion. The β_2-AR antagonist ICI-118551 inhibited the reduction suggesting the effect is via the receptor. Additionally stimulation with forskolin, producing a rise in cAMP similar to that caused by formoterol, decreased SERCA2 expression, suggesting that this is a cAMP dependent effect. Finally, pre-incubation with the ERK1/2 inhibitor U0126 blocked formoterol induced reduction of SERCA expression, indicating that ERK is involved in the signalling cascade. Under the same conditions the steroidal asthma treatment dexamethasone did not cause any change in SERCA (Ojo, 2011).

5.1.3 Diverging signalling cascades

β_2-AR receptors are desensitised by protein kinases to regulate and "dampen" signalling, an effect associated with an uncoupling of the receptor to its downstream mediators (Pierce *et al.*, 2002). Protein kinase A (PKA) was the first kinase shown to cause this response on the mammalian receptor (Benovic *et al.*, 1985), although the receptor can also be phosphorylated by another family of kinases called G-protein receptor kinases (GRKs) which unlike PKA is specific only for the constitutively active form of the receptor or agonist occupied form. As it only phosphorylates the active form of the receptor it is termed "homologous desensitisation" whereas PKA can induce "heterologous desensitisation" by phosphorylating a separate receptor to the one which activated it (Pierce *et al.*, 2002). Phosphorylation by GRKs can recruit β-arrestins to bind to the intracellular tail of the receptor, sterically hindering G-protein coupling and targeting it for internalisation. Subsequently, the receptor may undergo three processes: A) It is targeted to a recycling endosome where it is dephosphorylated and reinserted into the membrane, "resensitisation". B) It is targeted to a lysosome to be degraded, "down-regulation" (generally a consequence of prolonged signalling). C) It forms an intracellular signalosome in a complex with MAPK which is independent of the conventional G-protein mediated pathway (Shenoy *et al.*, 2006) and instead dependent on both β-arrestin-1 and 2. It has been shown that the agonists investigated here and used in asthma therapy, formoterol, salbutamol and salmeterol, all induce β-arrestin recruitment, although no signalling preference towards it, ERK phosphorylation or to the cAMP pathway has been observed in HEK293 cells (Drake *et al.*, 2008; Reiter *et al.*, 2012). Biased agonism is the term given to the phenomenon of receptors activating different

intracellular signalling pathways depending on the agonist that binds to them and their subsequent conformational change, reviewed in detail (Reiter *et al.*, 2012). The potential "imbalanced efficacy" elicited by differing downstream pathways therefore needs to be considered in this system where both the canonical cAMP and the β-arrestin dependent pathways are known to be activated (Fig 5.1.3-1).



5.1.3-1 Biased agonism in the context of the β_2-AR. The inactive form of the receptor can induce three different active conformations resulting in different signalling outcomes dependent upon their equilibrium constants and agonist association. It was previously believed that GPCRs activated secondary messengers in a linear fashion, new evidence suggests pathways are more likely arranged as integrated networks as described in this figure.

The downstream intracellular signalling pathway from the β_2-AR also has many points of divergence, one of which is immediately following a rise in cAMP. The discovery that Rap guanine nucleotide exchange factors were directly activated by cAMP independent of PKA opened the field of Epac1/2 research (Exchange protein directly activated by cAMP) (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Since it had been thought previously that PKA was the sole target of cAMP, the existence of Epac helped to explain previous data not truly fitting with the conventional model and further demonstrated the complexity of GPCR signalling.

A role for both Epac and PKA regulating ASM phenotype plasticity has been explored, with activation of both pathways shown to inhibit platelet derived growth factor induced modulation to a proliferative, hypocontractile phenotype (Roscioni *et al.*, 2011b). The mechanistic action of both agonists varied slightly as PKA stimulation inhibited both ERK and $p70^{s6k}$ phosphorylation whereas the Epac agonist only inhibited ERK (Roscioni *et al.*, 2011a). When epidermal growth factor was used it was only activation of the Epac pathway which inhibited the induced proliferation, as a PKA specific agonist had no effect (Kassel *et al.*, 2008). The previous reduction in SERCA2 expression by formoterol was shown to be dependent upon ERK activation as the inhibitor U0126 blocked the effect.

5.1.4 Aims and hypothesis

The aims of the experiments described in this chapter were therefore to delineate the intracellular signalling cascade from receptor activation to mRNA translation and also to determine whether this phenomenon occurs *in vivo*. Specifically, I hypothesised that intranasal dosing of formoterol in mice would cause a dose dependent reduction in ASM SERCA2 expression and that this would be mediated by the classical $G_{\alpha s}$ signalling cascade via cAMP activation.

An observation that *in vivo* treatment with LABAs does result in down-regulation of ASM SERCA2 expression could potentially explain the increased morbidity associated with long term use of β_2-AR agonists in asthma treatment. It was therefore of primary importance to test whether the observed effect of formoterol reducing SERCA2 expression *in vitro* translates to an *in vivo* setting.

5.2 Methods

5.2.1 Dosing formoterol *in vivo*

Balb/c mice were dosed intranasally (i.n.) once a day for four days before the lungs were harvested for SERCA2 quantification by western blot. Formoterol was dissolved in DMSO solution and diluted down to a $1mg.ml^{-1}$ stock in saline. Mice weighed on average 20g so 25µl of an $80µg.ml^{-1}$ stock was used for the $100µg.kg^{-1}$ group and 25µl of an $8µg.ml^{-1}$ stock for the $10µg.kg^{-1}$ group. Final concentration of DMSO was 0.8% v/v in 0.9% saline; this solution was used as the vehicle control. Mice were anaesthetised using isoflurane and the drug or vehicle control was added drop-wise to the nostrils for inhalation. Doses were based on previous i.n. dosing of formoterol (Wyss *et al.*, 2007).

5.2.2 Protein quantification

For whole lung protein quantification in the mice, lungs were excised, flushed with 0.9% saline and snap frozen in liquid nitrogen. Quantification of SERCA2 protein expression was performed by western blot as previously described in chapter 2.

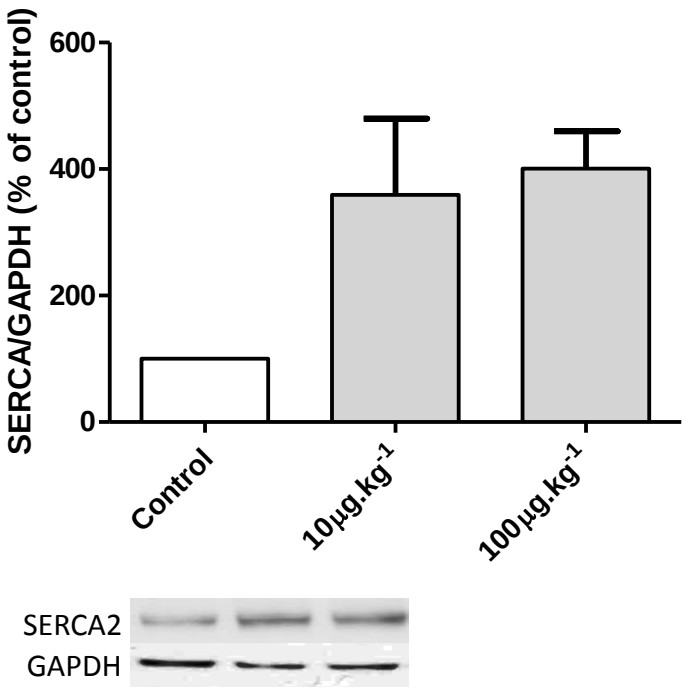
The following *in vitro* methods used in this chapter are also outlined in chapter 2 in the relevant sections.

5.3 Results

5.3.1 The effect of formoterol on SERCA2 expression *in vivo*

Formoterol caused an increase in SERCA2 expression which almost reached significance (p=0.06) by one-way ANOVA, with the apparent maximum effect occurring at a dose level of $100µg.kg^{-1}$ (Fig. 5.3.1-1). The experiment has low power and was therefore susceptible to a type 2 error; only a few mice were used

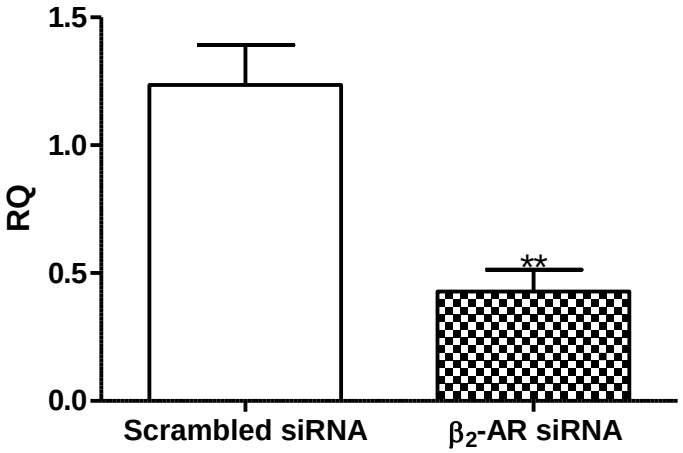
initially for a preliminary test in line with the three R's principles. As the result was opposite to expectation, I carried out additional *in vitro* experimentation before continuation.



5.3.1-1 Balb/c mice were dosed intranasally for four days with either 10 or 100µg.kg^{-1} formoterol. On day five the lungs were harvested for western immunoblot and SERCA2 protein levels measured and expressed as a percentage of the vehicle control. Bars represent mean ± SEM, n=3, p=0.06 by one-way ANOVA.

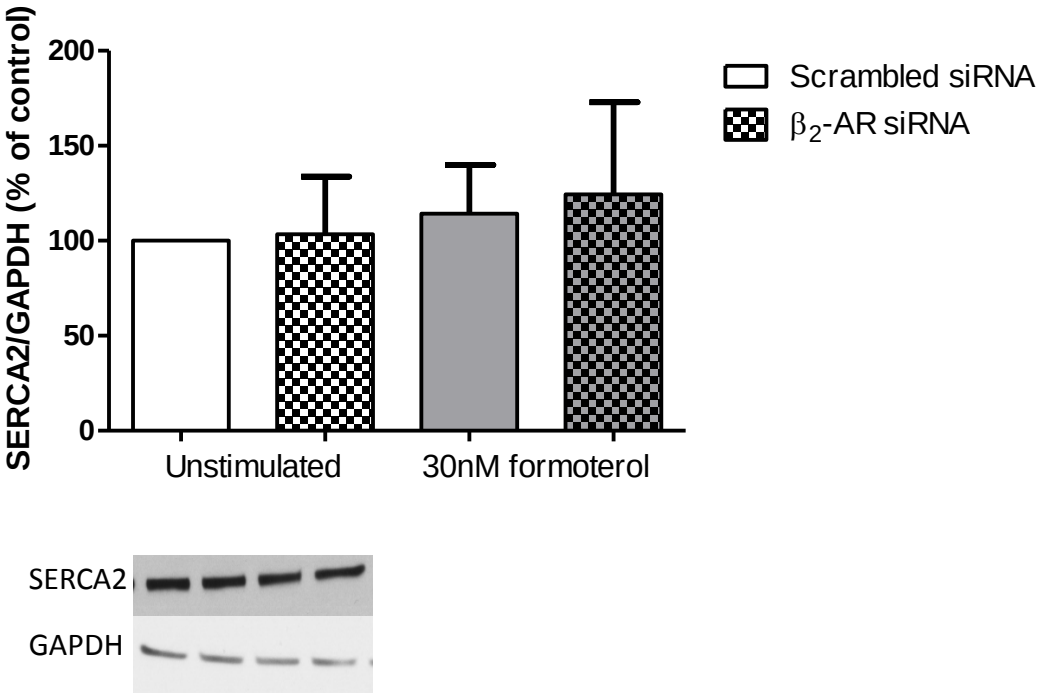
5.3.2 Clarification of β_2-AR involvement

To further clarify whether the observed *in* vitro effect involves the β_2-AR beyond use of pharmacological antagonism, an siRNA was used to knockdown the receptor via electroporation. Prior to this a control transfection was performed to confirm whether the siRNA was effective, with measurement of mRNA expression via qPCR as no suitable antibody for western blot was available (Fig. 5.3.2-1). Transfection with the β_2-AR siRNA resulted in a significant reduction in its mRNA compared to the scrambled control (0.42 ± 0.08 and 1.24 ± 0.16 relative quantity (RQ) respectively, p=0.01) confirming its efficiency and use for investigation with formoterol.

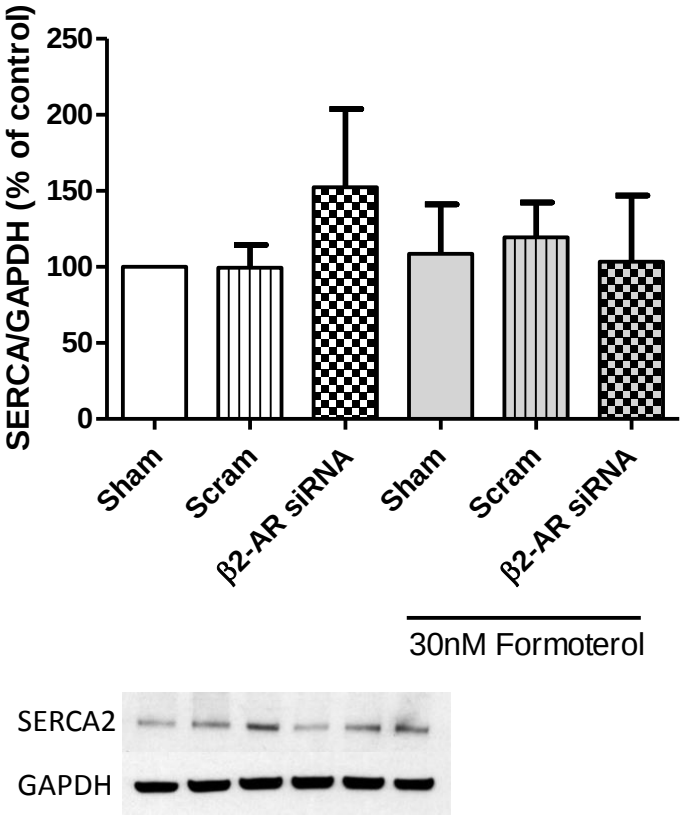


5.3.2-1 Healthy ASM cells were transfected with 250nM of β_2-AR targeted or scrambled siRNA. Cells were grown to confluence, RNA harvested and real-time PCR performed. Bars represent mean relative quantity (RQ), ± SEM, n=6. Analysed by unpaired t-test, p=0.01.

In contrast to Ojo's observation (Ojo, 2011), stimulation with 30nM formoterol failed to decrease SERCA2 expression as measured by western blot in the scrambled transfected group acting as the positive control (Fig. 5.3.2-2, p=0.88). No change was also observed in the β_2-AR transfected group (p=0.63). The lack of effect observed here led to the question that the transfection process itself may interfere with the results and affect SERCA2 expression. Therefore the experiment was repeated again performing a "sham transfection" whereby no RNA was introduced into the cells during the electroporation (5.3.2-3). Again, no decline in SERCA2 protein expression was observed following formoterol treatment in any of the three transfection groups. Surprisingly, transfection with the β_2-AR siRNA without formoterol stimulation resulted in an increase of SERCA2, although a definitive interpretation of these results is impossible owing to the small number of experiments which were carried out.



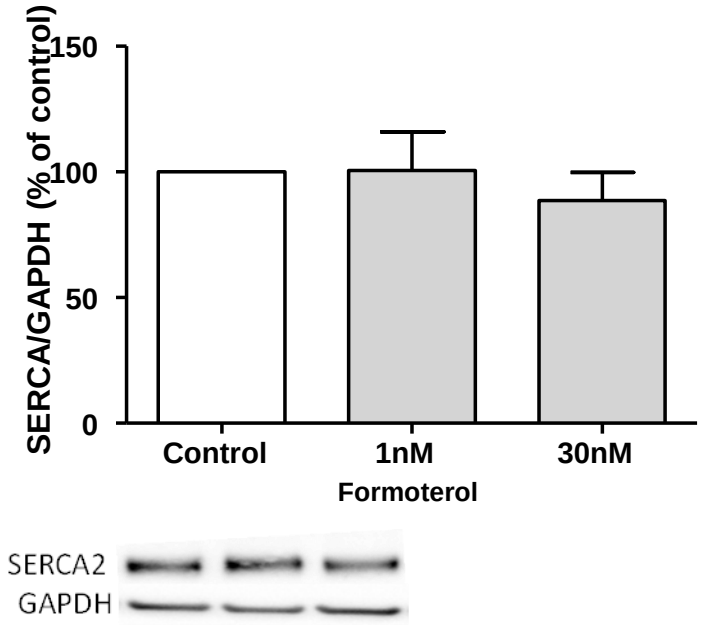
5.3.2-2 Healthy ASM cells were transfected with 250nM of β_2-AR targeted or scrambled siRNA. Upon reaching sufficient confluency cells were serum starved and treated with 30nM formoterol for 24 hours. Bars represent mean ± SEM, n=4.



5.3.2-3 Healthy ASM cells were transfected with 250nM of β_2-AR targeted, scrambled siRNA or deionised water. Upon reaching sufficient confluency cells were serum starved and treated with 30nM formoterol for 24 hours. Bars represent mean ± SEM, n=4.

5.3.3 Formoterol dose response

The lack of agreement between the preliminary data obtained previously in the lab and the results described above using the control siRNA made it necessary to re-investigate the original finding of formoterol causing a concentration dependent reduction in SERCA2 protein expression without the interference of electroporation. Fig. 5.3.3-1 questions the reproducibility of that finding as once again SERCA2 was not reduced by formoterol stimulation in healthy ASM cells. Whereas in the earlier experiments 30nM formoterol stimulation resulted in a significant reduction of SERCA2 after 24 hours incubation, I was not able to repeat this results in numerous different healthy derived cell lines.

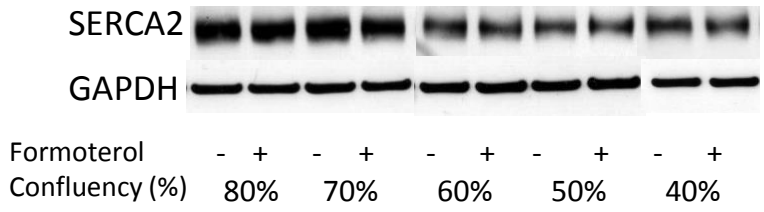


5.3.3-1 Healthy ASM cells were stimulated with either 1nM or 30nM formoterol for 24 hours. Changes in SERCA2 expression were measured by western immunoblot and expressed as a percentage of their un-stimulated control. Bars represent mean, ± SEM, n=6. One-way ANOVA; p=0.69.

5.3.4 Effect of cell density

It has been noted that the degree of confluency in cultured cells can affect their phenotypic properties, (see section 1.3.4). When cells are grown in culture they modulate from a contractile to a non-contractile state exemplified by a decrease in the contractile markers sm-MHC, calponin and desmin by up to 75% (Halayko *et al.*, 1996). Furthermore, l-caldesmon, vimentin, α/β-PKC and CD44 homing cellular adhesion molecule all increase one to six fold. The plasticity exhibited is bi-directional as once the "synthetic" cells start to reach confluence in culture the protein expression levels of smMHC and sm-α-actin increase back again (Halayko *et al.*, 1996). The implication of these findings is that the confluency of the cells dictate

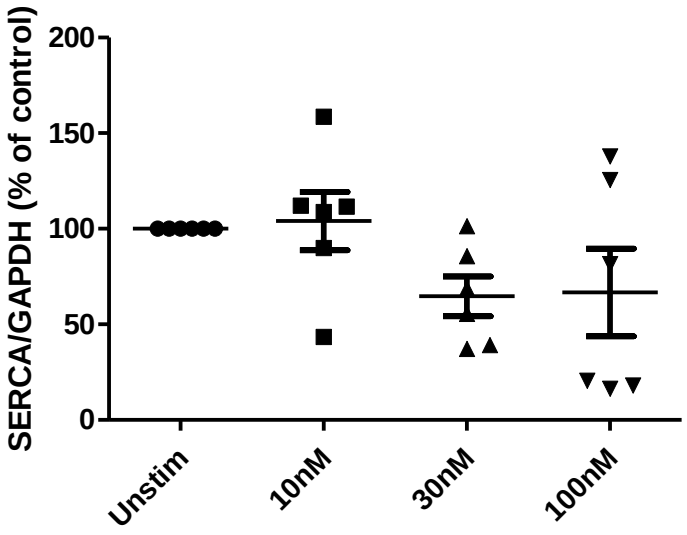
the expression of some markers, therefore stimulating cells consistently in this regard is essential to obtain consistent results. A disparity in cell confluence between investigators could underlie the lack of consistency. Therefore to test whether the degree of cell confluency alters the effect of formoterol on SERCA2 expression ASM cells were seeded at a range of confluences, serum starved and then treated with 30nM formoterol. A serial dilution was performed on the initial cell pellet to achieve an approximate range of confluency between 40-80% (although this was only qualitatively measured). Upon reaching the desired confluency cells were serum starved and subsequently treated with formoterol 72 hours later. During this serum deprivation period a further cell division usually took place so all of the approximations of confluency are slightly underestimated. Formoterol did not affect SERCA2 expression at any of the cell densities tested (Fig. 5.3.4-1, n=2), however a slight positive correlation was observed between SERCA2 expression and cell density, independent to treatment.



5.3.4-1 Healthy ASM cells were stimulated with 30nM formoterol for 24 hours. Changes in SERCA2 expression were measured by western immunoblot and expressed as a ratio of GAPDH expression. Bars represent mean, ± SEM, n=2.

5.3.5 The effect of salmeterol stimulation on SERCA2 expression

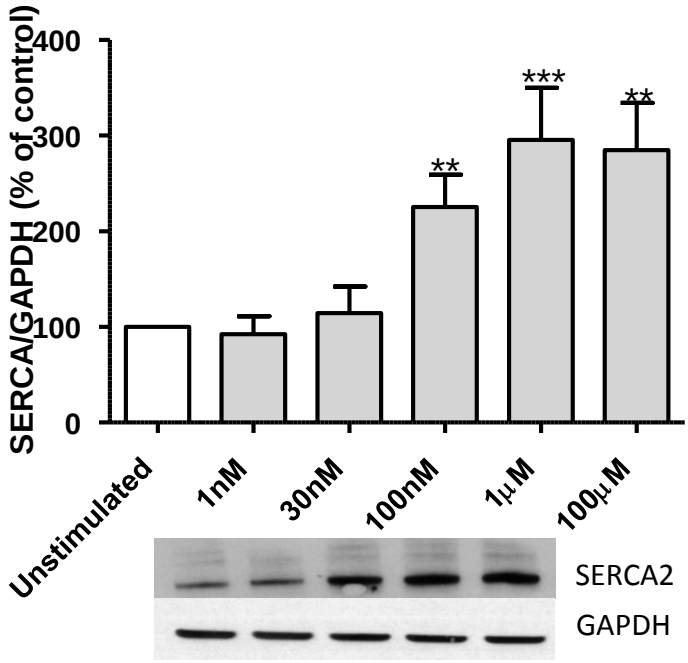
The original rationale for this chapter is based upon reports of the impact of long-acting β_2-AR agonists (LABAs) on mortality, and the speculation from others studies that a reduced ASM SERCA2 expression may be involved. To investigate whether this was an agonist specific or drug class phenomenon salmeterol was tested. In contrast to formoterol stimulation, salmeterol caused a concentration dependent decrease in SERCA2 expression. The effect was quite variable and did not reach significance ($p=0.08$, Fig. 5.3.5-1) and at the highest concentration, 100nM, the expression levels were reduced to 66.7 ± 23.0 % of control.



5.3.5-1 Healthy ASM cells were stimulated with salmeterol for 24 hours. Changes in SERCA2 expression were measured by western immunoblot and expressed as a ratio of GAPDH expression. Bars represent mean, ± SEM, n=6, p=0.08 by one-way ANOVA.

5.3.6 Effect of forskolin

In light of the discrepancy between formoterol and salmeterol reducing SERCA2 expression the intracellular signalling pathway was re-examined using forskolin to activate adenylyl cyclase, a downstream target of G_s following receptor activation. The experiment had been performed previously in the lab and was shown to reduce SERCA2 in line with previous formoterol data (Ojo, 2011). The concentrations of forskolin used in this previous experiment were 10µM and 100µM as they were found to induce a similar rise in cAMP as 10-30nM formoterol. However, my experiments demonstrated that forskolin caused a significant and concentration dependent *increase* in SERCA2 relative to GAPDH after 24 hours stimulation (Fig. 5.3.6-1, $p<0.0001$) with a maximal effect at 1µM (295.9 ± 54.3 % of control).

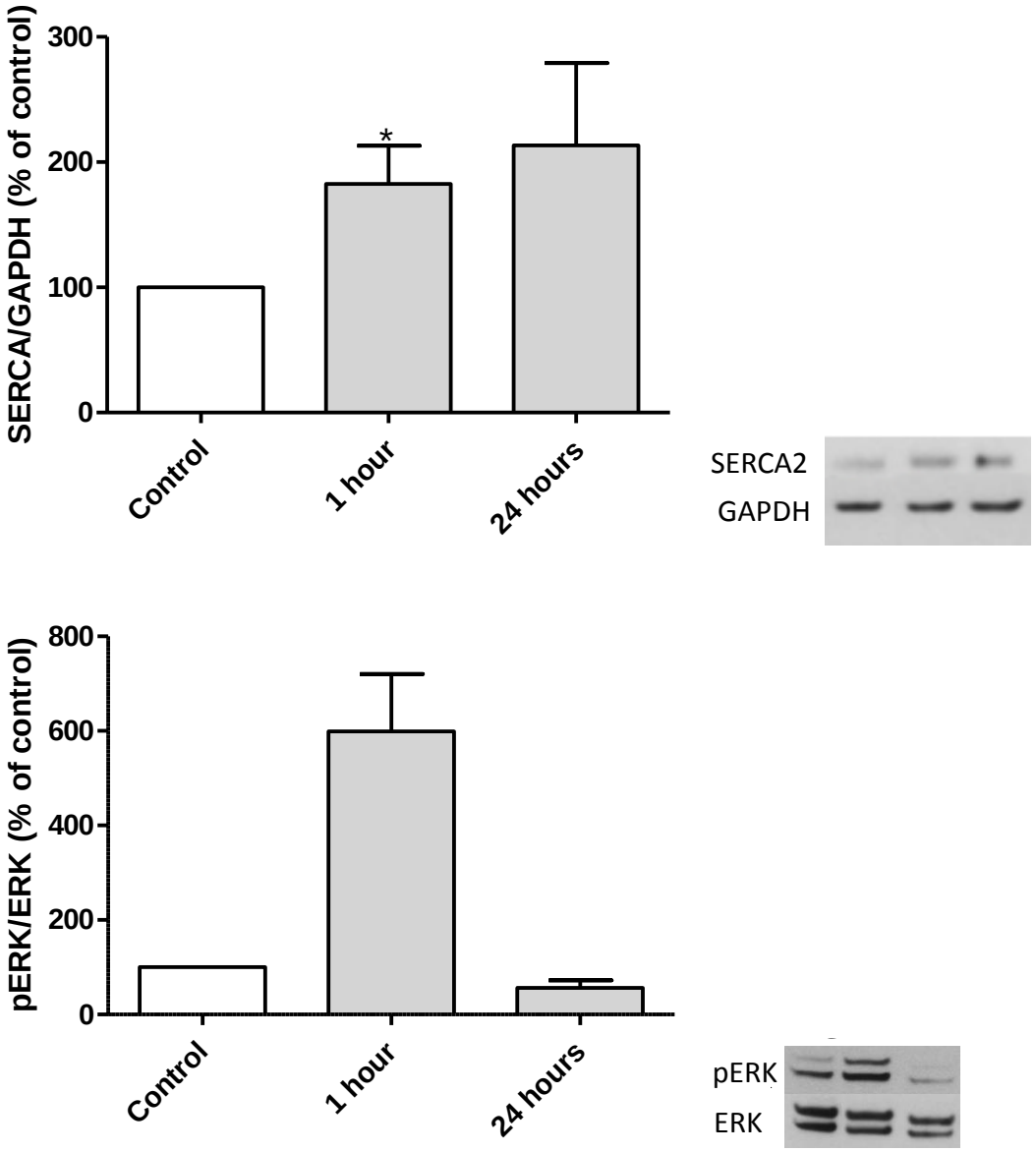


5.3.6-1 Healthy ASM cells were stimulated with a range of concentrations of forskolin for 24 hours. A) Bars represent mean ± SEM, n=3-8, $p<0.0001$ by one-way ANOVA and Bonferroni's multi comparison post test, **$p<0.01$, ***$p<0.001$.

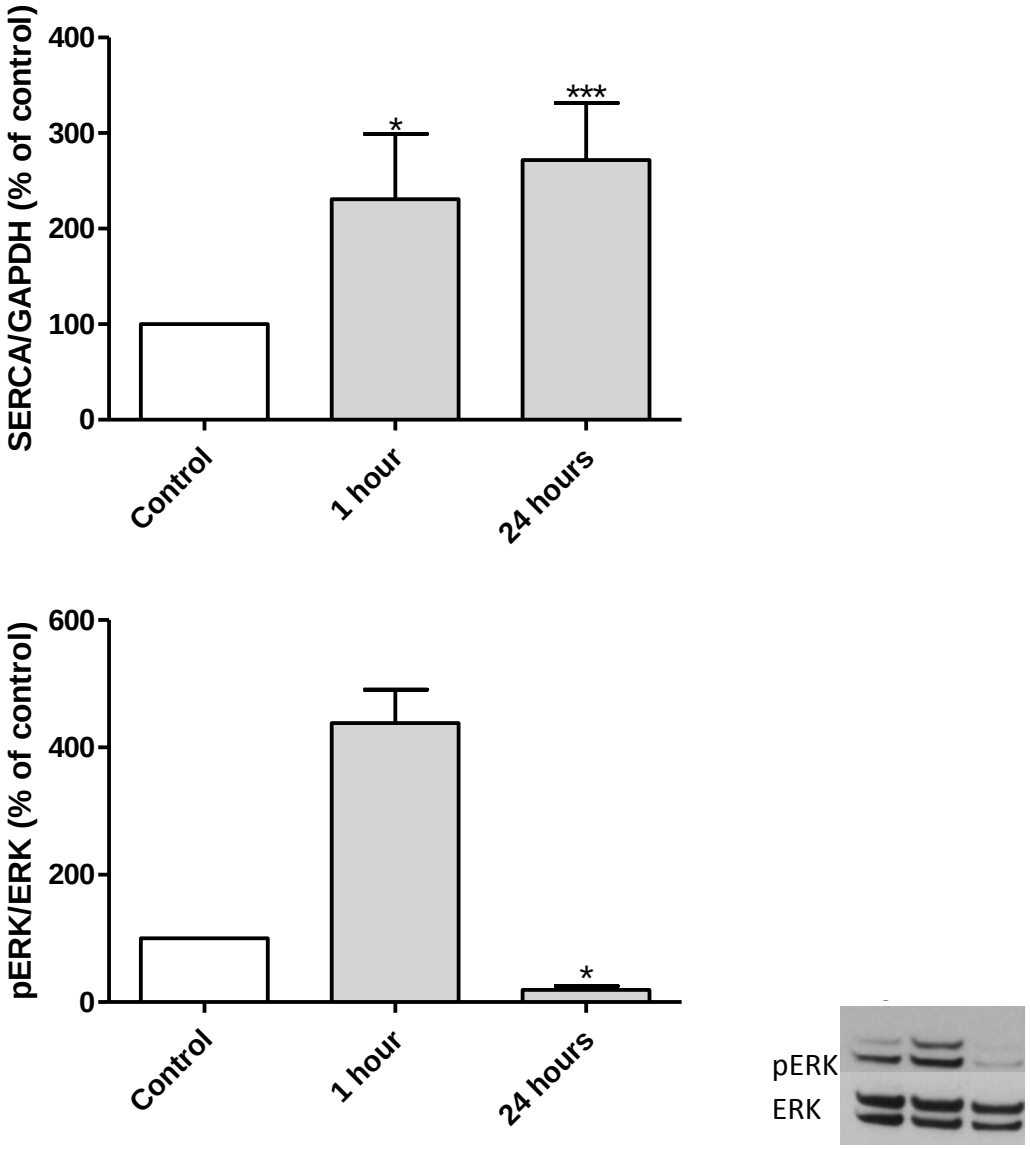
5.3.7 Post cAMP divergence of signalling

To delineate whether the cAMP mediated increase in SERCA2 expression was dependent upon PKA, Epac, or both, the specific agonists used in the previously mentioned publications were used. The protein kinase A (PKA) agonist 6-Bnz-cAMP (Biolog) is shown to be specific for PKA over Rap1 activation (Christensen *et al.*, 2003) and the Epac agonist 8-pCPT-2'-O-Me-cAMP (Biolog) doesn't activate PKA (Enserink *et al.*, 2002).

Stimulation with both the Epac and PKA specific agonists caused a transient increase in ERK phosphorylation rising at 1 hour post stimulation (598.7 ± 121.7% of control and 438.2 ± 52.5% of control, Figs. 5.3.7-1 and 5.3.7-2 lower respectively). Phosphorylation subsequently fell to below control levels at 24 hours post stimulation (56.2 ± 16.1% of control and 19.2 ± 6.1% of control, $p<0.05$ respectively). SERCA2 protein expression was also elevated by both agonists at both time points investigated. The Epac agonist significantly elevated SERCA2 at 1 hour post stimulation (182.5 ± 30.7% of control, $p<0.05$, Fig. 5.3.7-1) and protein expression further increased at 24 hours however this was not significant due to higher variance (213.5 ± 65.6 % of control). The PKA specific agonist significantly elevated SERCA2 protein expression at both the 1 hour time point (230.9 ± 68.4% of control, $p<0.05$) and at the 24 hour time point (271.8 ± 59.7% of control, $p<0.001$, Fig. 5.3.7-2).



5.3.7-1 Healthy airway smooth cells were stimulated with 100µM of the Epac agonist for up to 24 hours. Changes in A) SERCA2 expression or B) phosphorylated ERK were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean ± SEM, n=3-6, **p<0.01 by one-way ANOVA and Bonferroni's multiple comparison post test.

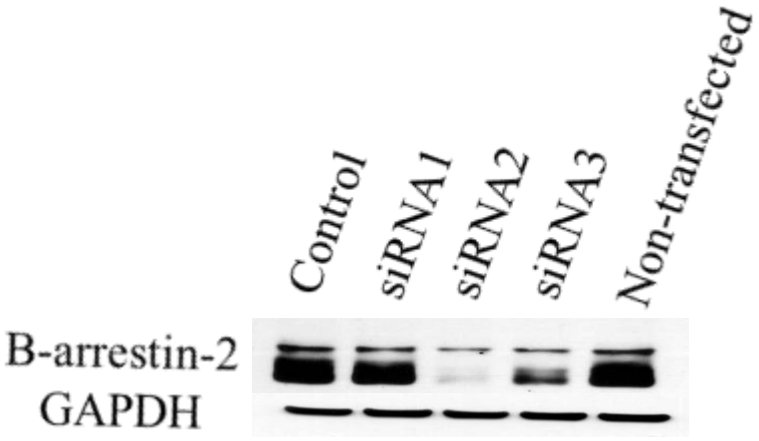


5.3.7-2 Healthy airway smooth cells were stimulated with 400µM of the PKA agonist for up to 24 hours. Changes in SERCA2 expression were measured by western immunoblot and expressed as a percentage of their unstimulated control. Bars represent mean ± SEM, n=3-8, by one-way ANOVA and Bonferroni's multiple comparison post test, *p<0.05, ***p<0.001.

5.3.8 The potential role of biased agonism

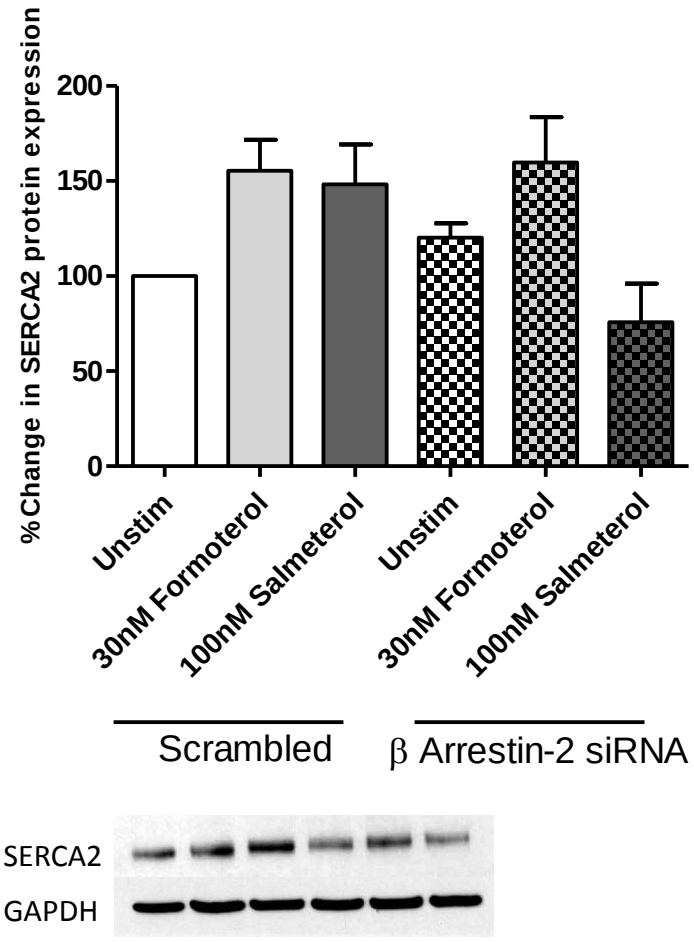
It has been shown that the β_2-AR is capable of activating both G-protein dependent signalling and β-arrestin-2 dependent pathways and that they can occur independently (Drake *et al.*, 2008; Reiter *et al.*, 2012; Shenoy *et al.*, 2006). The papers found no biased efficacy between salmeterol, formoterol and salbutamol in the cell systems they used however that does not rule out the possibility of an ASM cell specific or time dependent bias. Therefore the role of β-arrestin-2 signalling was investigated.

As elevation of cAMP by forskolin stimulation resulted in a robust increase in SERCA2 (Fig. 5.3.6-1), while salmeterol treatment caused a reduction (albeit variable, Fig. 5.3.5-1), an siRNA targeted against β-arrestin-2 was made to address whether this pathway might underlie the differences. A pool of three siRNAs were designed using the algorithm described in (section 2.6.1) and tested for efficiency (Fig. 5.3.8-1). The second siRNA of the pool resulted in the greatest knockdown so it was used in subsequent experiments.



5.3.8-1 Healthy ASM cells were transfected via electroporation with 3μg of three different siRNA sequences targeted against β-arrestin-2, along with a scrambled siRNA sequence and just the nucleofector solution alone as controls. Western blots were then carried out on the lysed cells to measure the efficiency of each siRNA construct, example blot shown.

The variable nature of the effect of salmeterol effect on SERCA2 expression prevented addressing the role of β-arrestin-2 in this pathway. The positive control (a reduction in SERCA2 expression using scrambled siRNA following salmeterol or formoterol stimulation) was not achieved. In fact a slight increase was observed, therefore the impact of reducing β-arrestin-2 expression could not be evaluated (Fig. 5.3.8-2).



5.3.8-2 Healthy ASM cells were transfected with either scrambled or β-arrestin-2 targeted siRNA and cultured in the presence or absence of 30nM formoterol or 100nM salmeterol for 24 hours. The ratio of SERCA2 protein expression over GAPDH was measured and normalised to the unstimulated scrambled transfected control. Data represents mean ± SEM, n=4.

5.4 Discussion

There is accumulating evidence to suggest that chronic use of long acting β_2-AR agonists leads to a physiological effect which diverges from their acute action, and may be detrimental. It was hypothesised at the beginning of this investigation that the increased mortality observed with formoterol use might, at least in part, be attributable to its dysregulation of calcium handling in ASM cells, thus driving them towards an asthmatic phenotype. It has been shown previously that a reduction in SERCA2 expression is observed in ASM cells derived from asthmatic patients compared to healthy patients and that artificially reducing expression can cause this switch in phenotype (Mahn *et al.*, 2009). The hypothesis was founded upon previous studies which had reported that formoterol caused a dose dependent reduction in SERCA2 expression (Ojo, 2011; Ryall *et al.*, 2008).

5.4.1 LABA administration *in vivo*

Our initial studies were thus designed to determine whether the data obtained from cultured ASM could be translated into an *in vivo* model, by treating mice with LABA and measuring SERCA2 expression in whole lung homogenates. However, contrary to the *in vitro* data, preliminary studies showed that treatment with formoterol apparently increased SERCA2 expression (Fig. 5.3.1-1). This unexpected finding cast serious doubt on the original hypothesis, although the study was limited in terms of physiological differences between mice and humans and use of homogenates of whole lung rather than ASM. In the light of the *in vivo* studies I therefore re-examined the effects of treatment with LABAs on SERCA2 expression in human cultured ASM.

5.4.2 An agonist specific effect?

Contrary to the studies of Ojo (2011), the data presented here suggest that salmeterol but not formoterol may reduce expression of SERCA2 in cultured healthy ASM cells (Fig. 5.3.5-1 and Fig. 5.3.3-1), but in a potentially cAMP-independent manner, as forskolin, a cAMP raising agent, increased SERCA2 expression (Fig. 5.3.6-1). cAMP activates two downstream pathways, via PKA and Epac (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Direct activation of either by use of reportedly specific agonists for PKA (Christensen *et al.*, 2003) or Epac (Enserink *et al.*, 2002) also caused an increased SERCA2 expression potentially mediated via ERK (Figs. 5.3.7-1 and 5.3.7-2). It has previously been reported that formoterol mediates its effect on SERCA2 expression in an ERK dependent manner (Ojo, 2011), so it would be of interest to see whether the cAMP mediated increase in SERCA2 expression also involves ERK. The data presented here showing a cAMP dependent increase in SERCA2 expression could be a further benefit to PDE inhibitors being developed for the treatment of asthma (see section 1.9.5). Both selective and dual inhibitors for PDE3/4 elevate cAMP to produce bronchodilation and anti-inflammatory effects, a rise in SERCA2 expression as shown here in the ASM will potentially contribute to both of these effects.

The difference in effect between the two LABAs poses an interesting conundrum which could shed light on the underlying process. Salmeterol is a partial agonist at the receptor yet reduced SERCA2 protein expression in a variable manner, on the other hand formoterol is a full agonist and had no discernable effect (Fig. 5.3.5-1 and Fig. 5.3.3-1 respectively) (Naline *et al.*, 1994). Another interesting difference is the possible enhanced attenuation in bronchodilation elicited by short-acting β_2-AR agonists by salmeterol compared to formoterol (van Veen *et al.*, 2003). As no biased agonism has been observed for either drugs (Drake *et al.*, 2008), and the data presented here shows cAMP increasing SERCA2 expression (Fig. 5.3.6-1) the answer could lie in the fact formoterol activates enough adenylyl cyclase to negate the β-

arrestin-2 dependent reduction in SERCA2. Whereas salmeterol as a partial agonist does not induce high enough levels of cAMP to maintain basal SERCA2 expression. Additionally the impact of cAMP reducing $[Ca^{2+}]_i$ cannot be ignored as it has been demonstrated in vascular smooth muscle cells that $[Ca^{2+}]_i$ and SERCA2b protein expression are positively correlated (Wu *et al.*, 2001). The relationship between cytosolic calcium concentration and SERCA2 expression with the results in this chapter lie in contrast as one would expect formoterol to lower calcium to a greater degree than salmeterol and hence additionally lower SERCA2. The relative impact of this effect could be dwarfed by that of the hypothetical β-arrestin-2 mediated effect.

5.4.3 A role for biased agonism?

As activation of β_2-AR caused a variable decrease in SERCA2, but its classical secondary messenger cAMP apparently had the opposite effect, it can be reasonably deduced that the receptor activates a secondary intracellular signalling cascade. The variable nature of the response to LABAs may therefore reflect changes in the balance between the cAMP pathway that enhanced SERCA2 expression and another that depresses it.

Classical receptor pharmacology theories have been largely revised in the last 15 years to accommodate evidence showing that different agonists acting on the same receptor can result in distinct signalling cascades by evoking multiple changes in receptor conformation (Drake *et al.*, 2008; Evans *et al.*, 2010; Reiter *et al.*, 2012). In particular, it is now known that phosphorylation of β_2-AR by GRKs leads to recruitment of β-arrestin and internalisation of the receptor; this is thought to be a key element of β_2-AR desensitisation in ASM (Deshpande *et al.*, 2008). It is believed that at low agonist receptor occupancy β_2-AR desensitisation is mediated by PKA and GRK independent, whereas at higher occupancy it is GRK/β-arrestin-2 dependent and PKA independent (Deshpande *et al.*, 2008; Wang *et al.*, 2009). In addition, internalisation by β-arrestin-2 can lead to the creation of a signalosome, independent to PKA activation, leading to G-protein independent activation of ERK (Shenoy *et al.*, 2006). The paper further demonstrates that ERK phosphorylation at early time points is PKA dependent however a switch to a β-arrestin-2 dependent pathway occurs after more prolonged exposure. It has been shown that all three of the agonists considered here (salbutamol, salmeterol and formoterol) are equally capable of activating either the cAMP or β-arrestin-2 dependent pathway in HEK293 cells (Drake *et al.*, 2008). I therefore hypothesised that the observed effects of the LABAs on SERCA2 expression in ASM could be related to a balance between cAMP and β-arrestin pathways.

To test this hypothesis, we designed a β-arrestin-2 siRNA and examined whether SERCA2 expression declined upon salmeterol stimulation in the control (i.e. scrambled transfected, vehicle stimulated group), but increased when β-arrestin-2 expression was suppressed, so that only the cAMP pathway was activated, as would be predicated by the hypothesis. The variable nature of salmeterol decreasing SERCA2 protein expression meant there wasn't a difference observed during the transfection controls, therefore no firm conclusions could be drawn with the siRNA (Fig. 5.3.8-2). The previous data showing that the effect of formoterol was dependent upon ERK activation (Ojo, 2011) could fit in with the β-arrestin-2 hypothesis as it too signals via it. Increasing the number of repeats might be able to provide a clearer picture in this response however the validity of the outcome would remain questionable due to the inherent variability. Therefore delineating the source of the variation observed and discrepancy between the findings of Ojo (2011) was addressed.

5.4.4 The influence of cell density

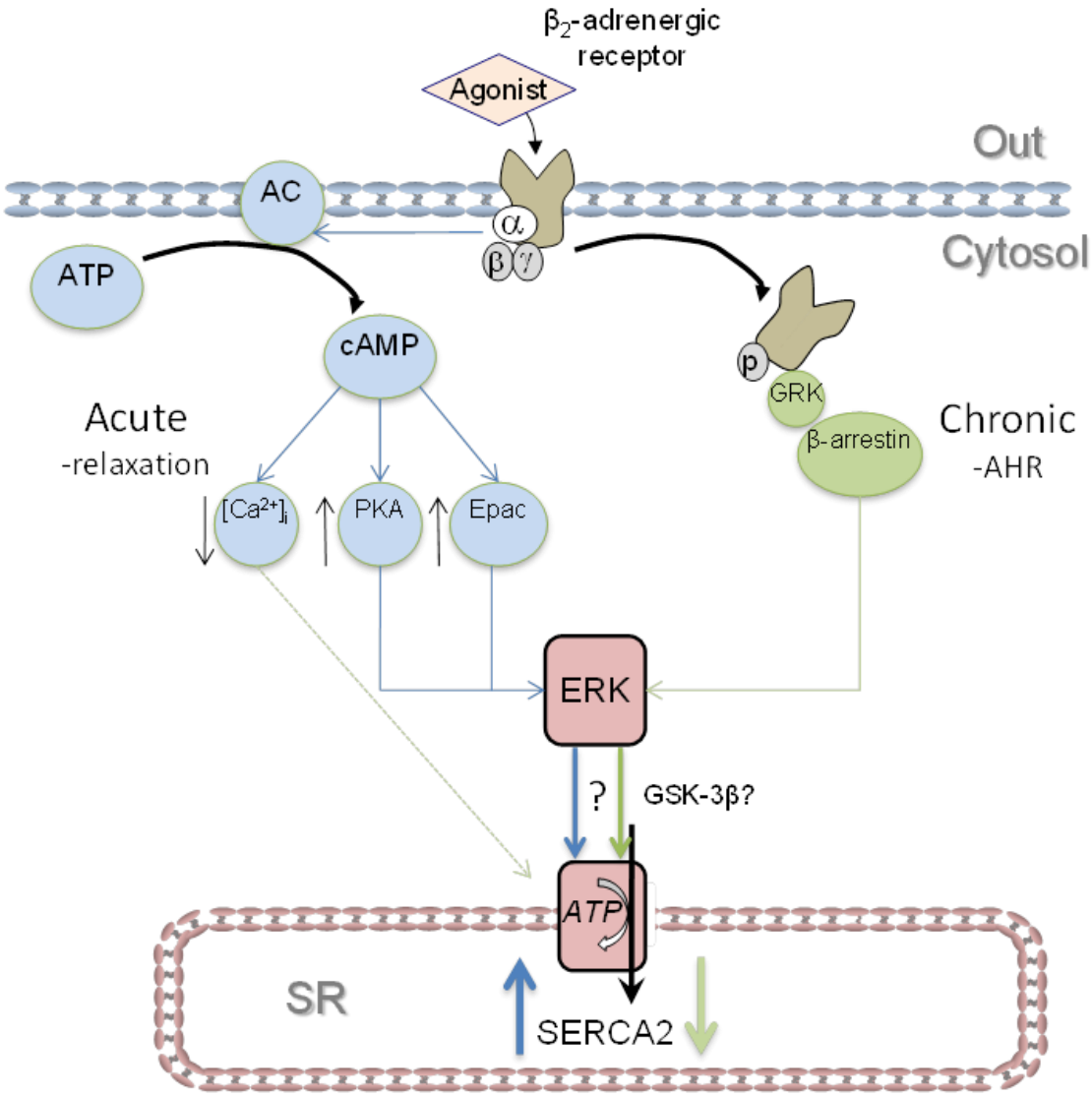
There is evidence that ASM phenotype changes as cells progress through to confluence. It has for example been shown that ASM cells at subconfluence express a modulated, proliferative phenotype which becomes contractile as they reach confluence (Halayko *et al.*, 1996). Furthermore, there is an increased smooth muscle bundle in the airways of asthmatics as a result of both hyperplasia and hypertrophy (Benayoun *et al.*, 2003; Chung, 2005; Ebina *et al.*, 1993). It has been postulated that a reduction in SERCA2 protein expression causing a higher intracellular basal calcium concentration contributes towards this hallmark of the disease (Mahn *et al.*, 2009). It is therefore plausible that SERCA2 expression is altered in differing cell densities as cells become more or less proliferative. Hence, the degree of confluence and rate of proliferation might explain the discrepancy between the data reported previously by Ojo (2011) and that shown here. Although the effect of formoterol did not change over a wide range of cell densities, the basal expression of SERCA2 in unstimulated cells appeared reduced in sparser cell populations (Fig. 5.3.4-1). The trend towards lower SERCA2 protein expression in the sparsely populated, more proliferative cells (Fig. 5.3.4-1) is consistent with the concept that the proliferative phenotype is associated with altered regulation of Ca^{2+} handling, which may drive this phenotype until confluence is achieved (Mahn *et al.*, 2010).

5.4.5 Conclusion

Due to the apparent variability of the response to LABAs, and our inability to discover the origin of this variability, the results of this study remain inconclusive. However, there is evidence that long term LABA treatment can be detrimental (Nelson *et al.*, 2006; Rodrigo *et al.*, 2009; Salpeter *et al.*, 2006), and that,

surprisingly, beta-blockers acting as inverse agonists may be beneficial in asthma (Hanania *et al.*, 2010; Hanania *et al.*, 2008; Nguyen *et al.*, 2009). In addition, the results shown here and by Ojo (2011) do suggest that β-agonists do have an effect of SERCA2 expression, although it is highly variable.

Therefore in spite of the inconclusive nature of these studies, I propose the following speculative model:



5.4.5-1 Stimulation of the β_2-AR on ASM cells may cause a dichotomy of response in a time dependent manner. Blue arrows indicate pathways increasing SERCA2 expression, green arrows decrease SERCA2 expression. Acutely the cAMP pathway is activated to control muscle tone and SERCA2 expression may be increased to improve the dynamics of signalling. Following chronic activation the receptor is targeted to β-arrestin-2 dependent signalosomes and decrease SERCA2 expression potentially inducing or enhancing an asthmatic phenotype (Michael *et al.*, 2004; Shenoy *et al.*, 2006). Both pathways are capable of signalling via ERK however its involvement in either or both phenomenon remains speculative.

Acute stimulation may result in cAMP elevation to control muscle tone and airway opening with the receptor being recycled back to the plasma membrane and chronic activation of the receptor leading to

down regulation and β-arrestin dependent gene expression changes. Higher levels of SERCA2 could be necessary in the contractile phenotype to ensure rapid reuptake of calcium and better defined calcium sparks for discrete temporal signalling (Fig. 5.3.4-1) whereas a decreased expression would lead to, and aid a proliferative phenotype by constitutively raised $[Ca^{2+}]_i$ (Abell *et al.*, 2011) driving ASM migration, secretion and proliferation (Mahn *et al.*, 2009; Mahn *et al.*, 2010) owing to the calcium dependence of the contractile machinery (Gerthoffer, 2008).

The increase in SERCA2 expression observed *in vivo* at the start of this chapter may be driven by the acute cAMP pathway and a longer dosing regimen, fitting more closely to the clinical setting, may drive the β-arrestin pathway and reduce SERCA2. The variability of the salmeterol-induced reduction of SERCA2 may reflect the cusp of switching between the two signalling pathways as some cell lines may respond at different rates. It would be of interest to stimulate at a 12 hour time point and for 1 week to see if the dichotomy is exposed.

Previous work in a murine model of asthma with a chronic dosing regimen of inverse agonists shows both a decrease in inflammation and mucous hyperplasia which were more pronounced than at earlier time points (Lin *et al.*, 2008; Nguyen *et al.*, 2008). Furthermore, β_2-AR null mice exhibited a similar beneficial effect and was shown to be essential for the full development of the asthmatic phenotype (Thanawala *et al.*, 2013). The results reflect the potential detrimental effects of activating the non-contractile β-arrestin-2 intracellular signalling cascade with current asthma therapies. The use of the inverse agonist propranolol as an asthma therapy suffered a recent setback as it showed no benefit over placebo in a double-blind trial in improving AHR or quality of life scores (Short *et al.*, 2013). The results lie in contrast with a previous open-label uncontrolled trial using nadolol where improvements in AHR were observed (Hanania *et al.*, 2008). The results demonstrate that biased agonism rather than inverse agonism at the β_2-AR may be the solution to the current reported problems or more careful selection of the patient sub-group/inverse agonist is necessary (Kazani *et al.*, 2013).

In conclusion a mainstay therapy target for asthma, the β_2-AR can modulate the expression of SERCA2 in ASM cells. SERCA2 reduction has been shown to induce an asthmatic phenotype which may help explain the chronic detrimental effects associated with this drug class. The manner in which this is done and the clinical importance of this effect still need to be elucidated but a time-dependent switch in downstream receptor signalling could play a role.

Chapter 6 Characterising an immortalised airway smooth muscle cells characterisation

6.1 Introduction

Currently, the vast majority of research performed *in vitro* on ASM cells utilises primary cell lines derived from patients with varying disease severities, and grown in culture for a maximum of three to six passages. This is because of the very limited amount of tissue available from biopsies, and the need to enhance tissue mass for most forms of experimentation. While such cells can provide an invaluable insight into disease mechanisms, they can be misleading due to phenotypic drift during multiple passages (see 1.3.4).

6.1.1 Immortalisation

To circumvent this problem we have taken ASM cell lines derived from healthy, mild or moderate asthmatics, and in collaboration with Professor Andrew Halayko's group in Winnipeg, Canada, attempted to create phenotypically stable, disease-specific immortalised cell lines. The initial six ASM cell lines investigated in this chapter were sent from our department to Winnipeg for immortalisation, though others are ongoing. The hTERT (human telomerase reverse transcriptase) gene was introduced into the cell genome by random insertion using a MMLV-based retroviral vector (Moloney murine leukaemia virus). Telomerase serves to maintain chromosomal integrity by capping DNA strands with tandem hexameric repeats ending in a 3' single strand overhang (Daniel *et al.*, 2012). In somatic cells telomerase remains inactive and as every replication of DNA is incomplete the strand gradually shortens until senescence occurs. Dysregulation of telomerase transcription is a critical step in human carcinogenesis enabling the capacity for tumours to progress (Kyo *et al.*, 2008). By taking advantage of this and expressing telomerase to high levels in somatic cells they can effectively become immortalised by never reaching senescence via repeated DNA replication.

The populations produced from the transductions are heterogeneous, made up of many colonies from independent insertion events. The heterogeneity should have a "normalising" effect on the cell lines, correcting internally for any insertion-related side effects. Although any insertions into critical genes should be normalised by the expected majority of insertion events into introns, there is a reasonable likelihood that subtle changes in phenotypes could occur that would not be masked by the population. Due to the nature of the immortalisation it is essential to characterise the cells to ensure their disease-state-specific phenotype is maintained.

6.1.2 Cell lines versus primary cells

There are numerous additional advantages to creating a set of disease-specific immortalised ASM cell lines. The use of siRNAs has been demonstrated previously in this chapter using transfection with plasmids. An immortalised ASM line would negate the need for multiple transfections and allow the creation of permanent over-expressing or knockout cell lines, saving both time and money. Recruitment of patients to continually get tissue via bronchoscopy requires several staff, use of hospital core facilities and a large investment of time and money. None of which would be required once characterised lines are established and research could be carried out without access to the infrastructure of a hospital. Finally, less variation in experimental data would be expected from using the same lines repeatedly. Asthma is a heterogeneous disease and ASM cells derived from the same patient sub-sets also show variability due to both genetic and environmental factors. Using the same (well characterised) immortalised cell line for investigating intracellular signalling pathways for example would be of great use as the reduction in variability would lead to much higher experimental power when investigating a hypothesis. An inherent problem with this is that it would be much less representative of the population as a whole so care would be needed when extrapolating data acquired to the clinical setting.

The methodology in this chapter was broadly based on a previous study from our lab showing that protein expression of SERCA2 in ASM cells is negatively correlated to asthma disease severity (Mahn *et al.*, 2009). Furthermore, the paper shows a decreased $[Ca^{2+}]_i$ peak response following bradykinin stimulation and a longer recovery time to baseline demonstrating a smaller store content and filling because of less SERCA2. Possibly as a further result of decreased SERCA and hence the potentially increased basal $[Ca^{2+}]_i$, an increase in eotaxin-1 production following IL-13 stimulation and proliferation is also observed (Mahn *et al.*, 2009). These indices were used as a benchmark to discover whether the immortalised ASM cell lines obtained have retained these asthmatic phenotypes. The data included here were part of a preliminary study during the development phase, and there were a number of limitations.

6.1.3 Aims

To this end, the aim of this chapter was to discover the extent each cell line investigated fits with previously published phenotypic differences between healthy, mild and moderate cultured ASM cells. The criteria assessed for each line was:

- Proliferation – measured by tritiated thymidine incorporation and the MTT assay.
- Eotaxin-1 secretion following IL-13 stimulation.

- Calcium mobilisation following bradykinin stimulation.
- SERCA2 protein expression.
- Smooth muscle marker expression.

Originally all of the data collected was going to be compared back to data collected from their respective native lines. Such a comparison would have shown how much the cells were altered by the hTERT insert or whether they still represented their native state. The native lines that returned with their immortalised counterparts were at a too high passage to grow; therefore these comparisons could not be made. This is being addressed for our current studies.

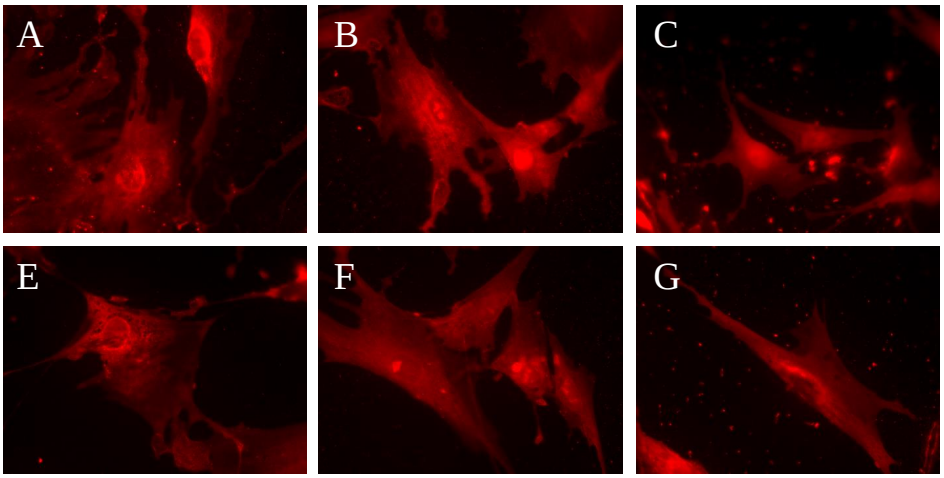
6.1.4 Hypothesis

I hypothesised that following immortalisation most of the cell lines would respond in line with the data described in (Mahn *et al.*, 2009). Conversely as only single lines were investigated and only two from each disease severity, the correlations would not be as clearly defined as previously published owing to the natural variation in the parameters assessed.

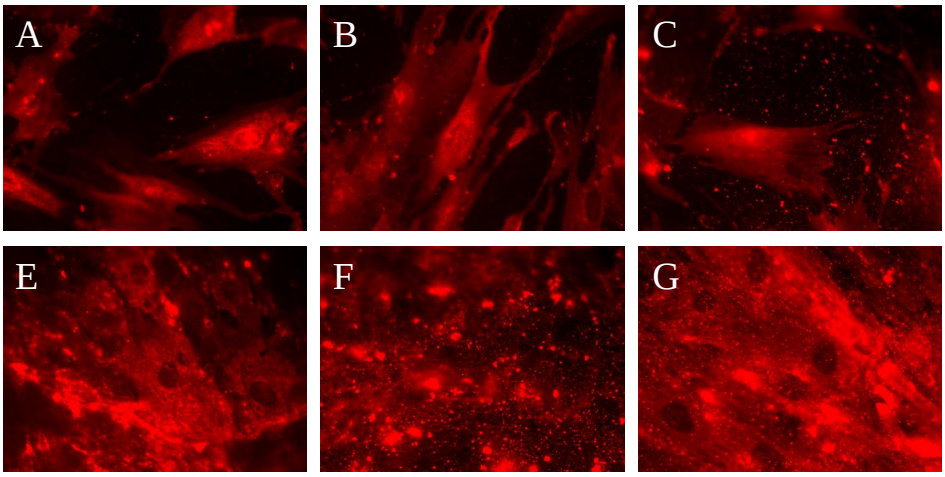
6.2 Results

6.2.1 Smooth muscle markers

Firstly, the cells had to be confirmed as ASM cells, so they were stained for the typical ASM markers smooth muscle α-actin, desmin and calponin (Halayko *et al.*, 1996) by immunocytochemistry. Fig. 6.2.1-1 shows some example staining from the healthy immortalised cell line htHB96 and the moderate asthmatic-derived immortalised cell line htHB107 at a low passage. All of the immortalised cells were positive for the markers tested. To ensure they maintained their markers after a significant time in cell culture staining was repeated after ten passages (Fig. 6.2.1-2). Once again all of the cells stained positive. The quality of the staining in the cell line htHB107 deteriorated at the latter passages possibly due to secondary antibody issues.



6.2.1-1 Staining for ASM cell markers; sm-α actin (A&E), desmin (B&F) and calponin (C&G). Cells in pictures A-C are derived from the healthy patient htHB96 and E-F from the moderately asthmatic htHB107. Pictures were taken no more than three passages following immortalisation.



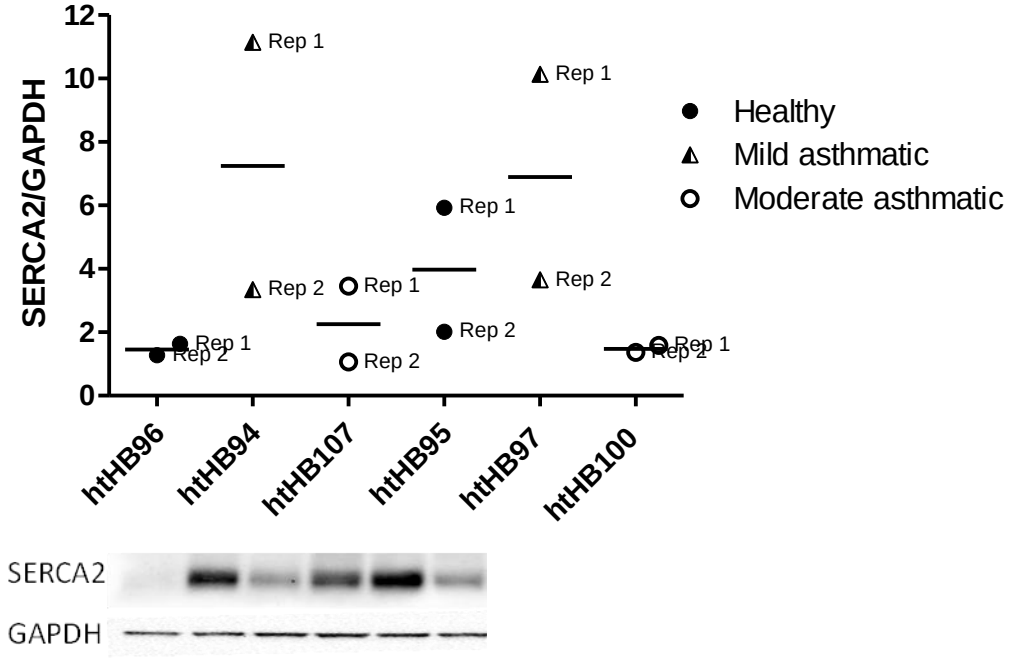
6.2.1-2 Staining for ASM cell markers; sm-α actin (A&E), desmin (B&F) and calponin (C&G). Cells in pictures A-C are derived from the healthy patient htHB96 and E-F from the moderately asthmatic htHB107. Pictures were taken ten passages following immortalisation.

6.2.2 SERCA expression

The central theme to (Mahn *et al.*, 2009) shows a negative correlation between SERCA2 protein expression and asthma severity. Here the protein expression of SERCA2 normalised to GAPDH provided mixed results (Fig. 6.2.2-1). The western blots were ran in duplicate with separate cells from the same line to minimise erroneous results. The occasion of the repeat (Rep 1 or Rep 2) is listed on the graph because on first site there appears to be large variation however the same expression pattern is observed both times.

Both htHB94 and htHB97 derived from mild asthmatics had much higher relative levels of SERCA2 compared to the other cell lines (mean 7.24 and 6.90 arbitrary units (AU) respectively). The healthy cell

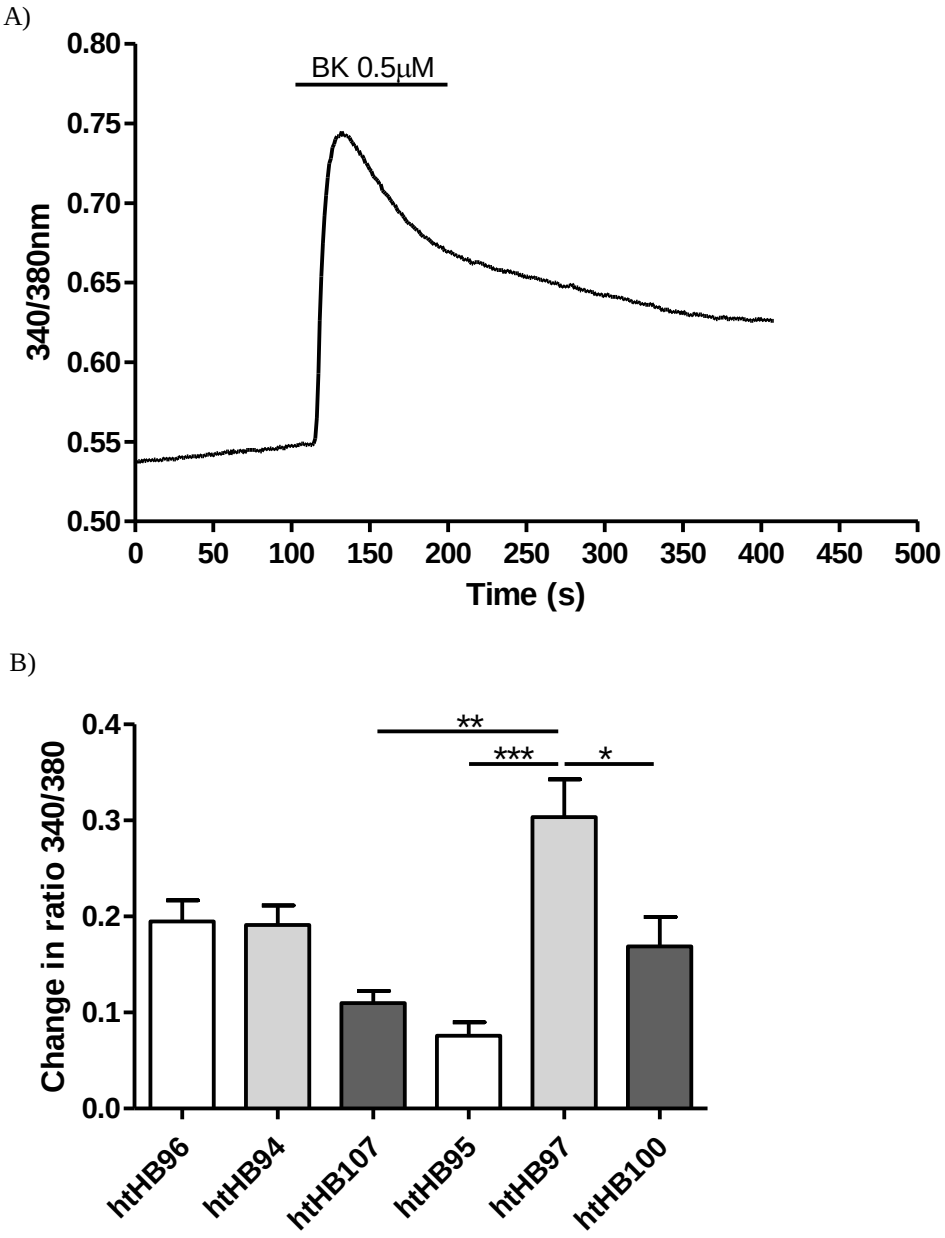
line htHB95 had higher levels (3.97 AU) than the two moderate asthmatic lines htHB100 and htHB107 (1.47 and 1.85 AU respectively) but contrary to previously published results it had lower expression than the mild asthmatic lines. The final healthy cell line htHB96 had much lower than expected SERCA2 expression (1.46 AU). Between the cell lines there was no clear correlation between disease severity and SERCA2 expression in these tested immortalised ASM cell lines.



6.2.2-1 Immortalised ASM cells were grown to confluence, lysed and SERCA2 protein levels measured by western blot. Cell lines were either derived from healthy patients (white), mild asthmatics (light grey) or moderate asthmatics (dark grey). Bars represent mean in duplicate.

6.2.3 Calcium release data

To test the functional impact of any change in SERCA2 expression the sarcoplasmic reticulum stores were partially emptied with the agonist bradykinin. The peak rise in $[Ca^{2+}]_i$ is indicative of store content and the rate of recovery back to baseline is in part dependent upon SERCA2 activity. Although the peak rise in $[Ca^{2+}]_i$ following bradykinin stimulation did not correlate well with measured SERCA2 levels they did fit more closely with previously published data. The healthy cell line htHB96 had a larger rise in $[Ca^{2+}]_i$ compared to the line htHB107 derived from a moderate asthmatic (0.19 ± 0.02 Δ340/380nm and 0.11 ± 0.01 Δ340/380nm respectively). The large response in htHB97 which was significantly greater than the moderate asthmatic lines htHB100 and htHB107 as well as the healthy line htHB95 ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) only partially reflects the predicted pattern expected from the disease state however it does correlate best with its measured SERCA2 expression.

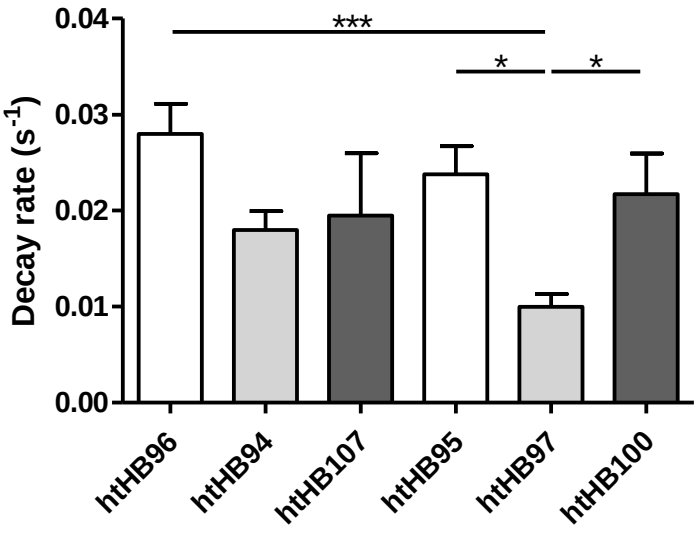


6.2.3-1 Functional consequences of stimulating immortalised human ASM cells with 0.5µM bradykinin. Cells were perfused with HBSS to achieve a steady base-line then stimulated with bradykinin, the peak ratiometric measurement (340/380nm) indicating calcim store release was recorded. A) A representative calcium transient trace. B) Cell lines were either derived from health patients (white), mild asthmatics (light grey) or moderate asthmatics (dark grey). Bars represent mean ± SEM, (n=3-9). Analysis by one way ANOVA and Bonferroni's post test; *p<0.05, **p<0.01, ***p<0.001.

6.2.3.1 Decay rate of intracellular calcium following bradykinin stimulation

The recovery rate of $[Ca^{2+}]_i$ returning back to a steady state following bradykinin stimulation and release from stores was used to indicate SERCA2 activity (Fig. 6.2.3-2). As with the calcium release data (Fig. 6.2.3-1) the cell line htHB97 provided the only source of significant statistical variation as analysed by a one way ANOVA and Bonferroni's post-test from the other cell lines. Its rate of recovery was

significantly slower compared to the healthy lines htHB96 and htHB95 as expected ($p<0.001$ and $p<0.05$ respectively) but also slower than htHB100 derived from a moderately asthmatic patient ($p<0.05$).



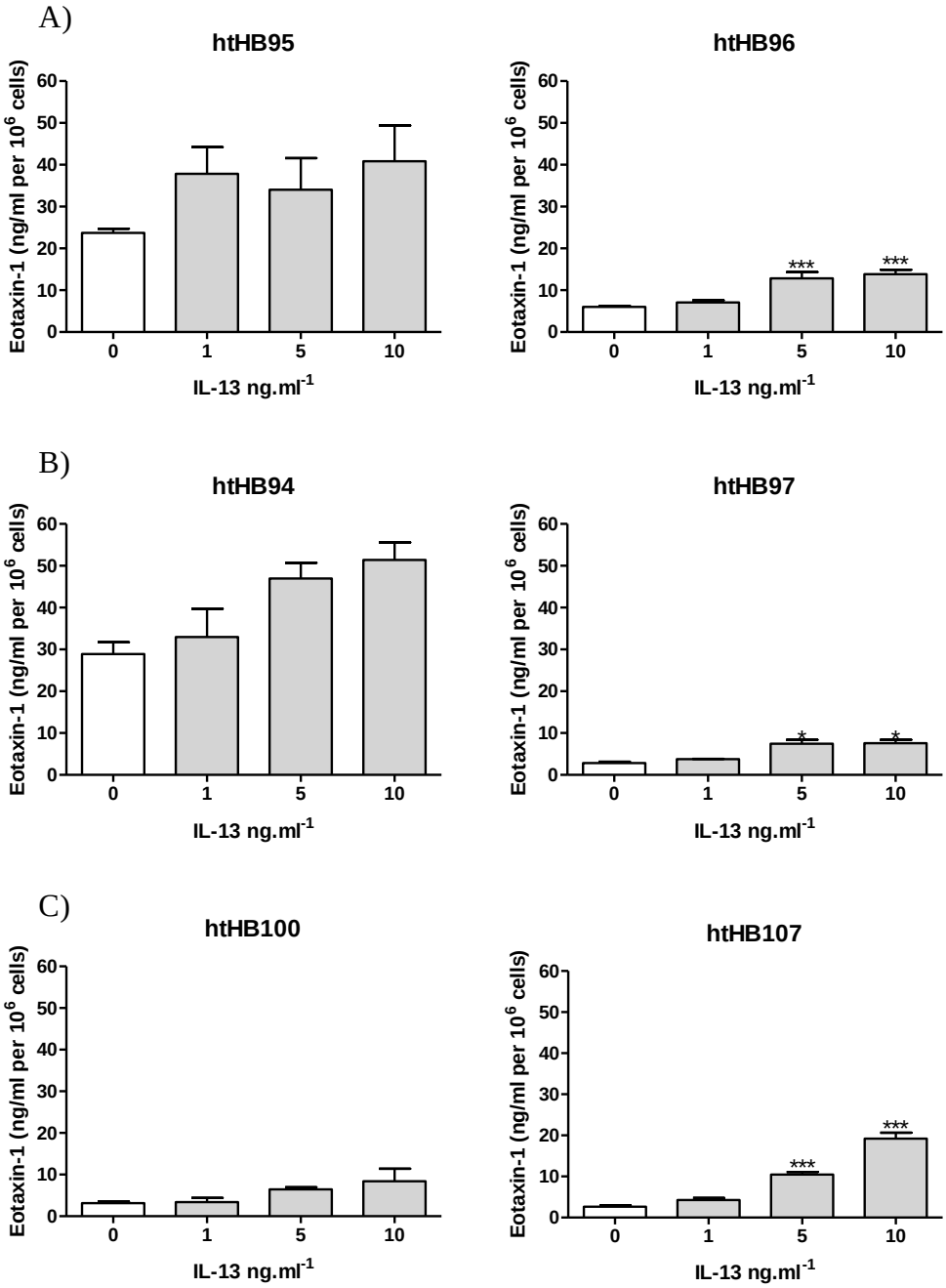
6.2.3-2 The rate of stimulating immortalised human ASM cells with 0.5μM bradykinin. Cells were perfused with HBSS to achieve a steady base-line then stimulated with bradykinin. A single, 3 parameter exponential decay line was fit from the peak ratiometric measurement (340/380nm) to the baseline with the gradient indicating rate of re-uptake. A) A representative calcium transient trace. B) Cell lines were either derived from health patients (white), mild asthmatics (light grey) or moderate asthmatics (dark grey). Bars represent mean ± SEM, (n=2-9). Analysis by one way ANOVA and Bonferroni's post test; *p<0.05, **p<0.01, ***p<0.001.

6.2.4 Eotaxin-1 release

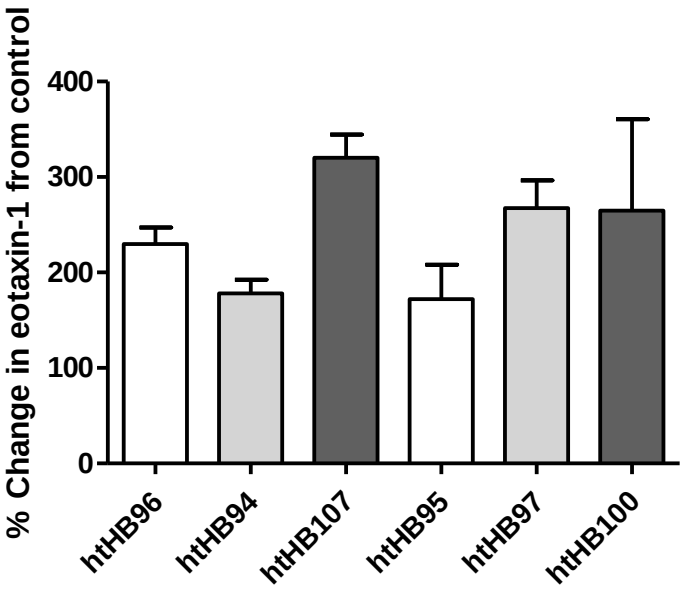
There are three different isoforms of eotaxin; 1-3 all involved in the chemotaxis of eosinophils. Eotaxin-1 is secreted from ASM cells and its levels, along with its receptor CCR3 have been reported to be increased in asthma and increase ASM migration *in vitro* (Joubert *et al.*, 2005), this effect is positively correlated with disease severity (Pepe *et al.*, 2005). The T_H2 cytokine IL-13 is also raised in asthma (see 1.2.2) and has been shown to augment the release of eotaxin-1 (Mahn *et al.*, 2009) which is further enhanced with the co-culture of asthmatic derived ECM proteins (Chan *et al.*, 2006).

All of the cell lines released an increasing amount of eotaxin-1 in a concentration dependent manner to IL-13 stimulation. Comparing the eotaxin-1 release as a percentage of the unstimulated control (Fig. 6.2.4-2) shows that in the case of both healthy and both moderately severe derived ASM cell lines an asthmatic phenotype increases the amount of eotaxin-1 production for a given amount of IL-13 exposure, however there were no statistically significant differences between the lines. The smallest increases in eotaxin-1 production from the unstimulated controls were observed in the cell lines htHB95 and htHB94 (172.3 ± 36.0 and 178.1 ± 14.39 % of control respectively) which also displayed the highest basal secreted levels

(23.7 ± 1.0 and 28.9 ± 2.8 ng.ml^{-1} per 10^6 cells respectively, Fig. 6.2.4-1). The moderately severe derived line htHB107 saw the biggest and most robust increase in eotaxin-1 upon 10ng.ml^{-1} IL-13 stimulation compared to basal release (320.1 ± 24.4 % of control, $p<0.001$). The cell lines htHB96 and htHB97 also both released a significantly greater amount of eotaxin-1 following 10ng.ml^{-1} IL-13 stimulation ($p<0.001$ and $p<0.05$ respectively).



6.2.4-1 Immortalised ASM cells were grown, serum starved for 72 hours and stimulated with varying concentrations of IL-13 or vehicle control. The cell density was calculated, the cells then lysed and eotaxin-1 concentration measured by ELISA. Cell lines were either derived from A) healthy patients, b) mild asthmatics or C) moderate asthmatics. Bars represent mean ± SEM, n=2-6 (in duplicate); *$p<0.05$, $p<0.01$ and ***$p<0.001$ as measured by one way-ANOVA and Bonferroni's multi comparison post test.



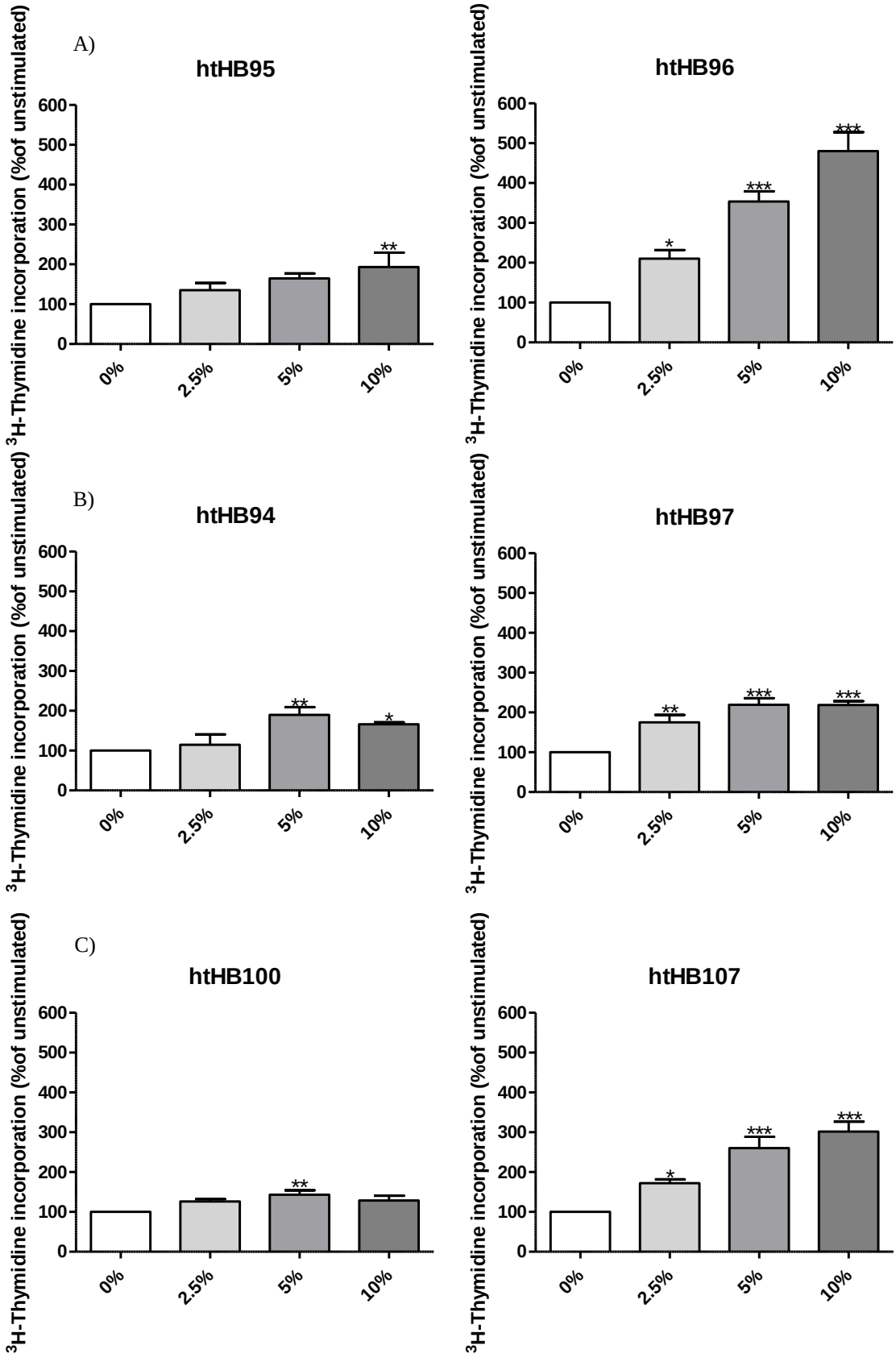
6.2.4-2 Immortalised ASM cells were grown, serum starved for 72 hours and stimulated with varying concentrations of IL-13 or vehicle control. The cell density was calculated, the cells then lysed and eotaxin-1 concentration measured by ELISA. Cell lines were either derived from healthy patients (white bars), mild asthmatics (light grey) or moderate asthmatics (dark grey). Bars represent mean ± SEM, n=2-6 (in duplicate).

6.2.5 Proliferation

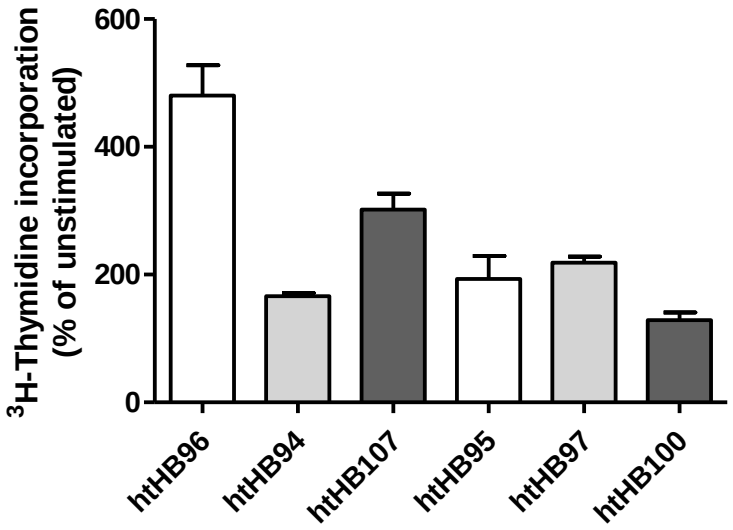
6.2.5.1 ^{3}H-thymidine

It has been well documented that there is increased ASM mass in the airways of asthmatics due to both hyperplasia and hypertrophy and cultured cells from asthmatic patients retain this enhanced proliferative phenotype (Johnson *et al.*, 2001). It has been hypothesised that the increased proliferation maybe a result of the decreased SERCA2 expression in asthmatic ASM cells as treatment with a targeted siRNA in healthy cells recapitulated the effect (Mahn *et al.*, 2009). Here the tritiated thymidine incorporation assay is used to measure cellular proliferation as it is a direct indication of DNA synthesis.

Every cell line measured showed an increase in proliferation with higher concentrations of FBS (Fig. 6.2.5-1). Analysing the cells stimulated with 10% FBS as a percentage of the unstimulated controls revealed a weak, negative correlation between proliferation and disease severity contrary to previously published data and theory (Fig. 6.2.5-2). The cell line htHB107 showed a significantly greater increase in proliferation in response to 10% FBS compared to the healthy line htHB95 (301.8 ± 25.2 and 193.3 ± 35.9% of control respectively, p=0.02).



6.2.5-1 Immortalised ASM cells were grown, serum starved for 72 hours and stimulated with varying concentrations of FBS for 4 hours. The cells were then pulsed with 3.7 Bq of tritiated thymidine and left to grow for a further 48 hours before being lysed and quantified using a beta counter. Cell lines were either derived from A) healthy patients, b) mild asthmatics or C) moderate asthmatics. Bars represent mean ± SEM, n=6-12; *p<0.05, p<0.01 and ***p<0.001 as measured by one way-ANOVA and Bonferroni's multi comparison post test.

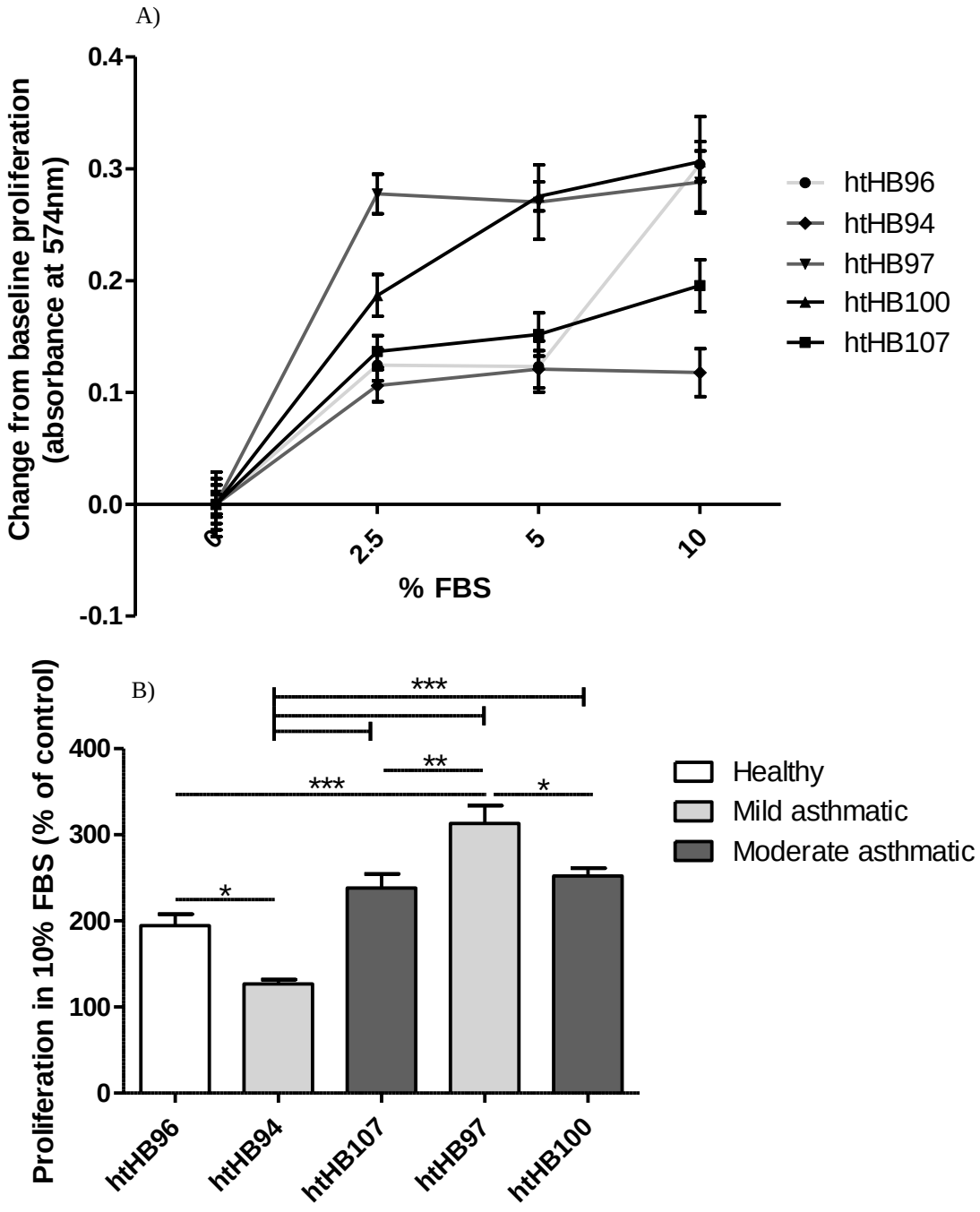


6.2.5-2 Immortalised ASM cells were grown, serum starved for 72 hours and stimulated with varying concentrations of FBS for 4 hours. The cells were then pulsed with 3.7 Bq of tritiated thymidine and left to grow for a further 48 hours before being lysed and quantified using a beta counter. Cell lines were either derived from healthy patients (white bars), mild asthmatics (light grey bars) or moderate asthmatics (dark grey bars). Bars represent mean ± SEM, n=6-12.

6.2.5.2 MTT assay

Due to the unexpected proliferation results from the tritiated thymidine assay, thought of as the "gold standard" in proliferation testing, an MTT assay was performed as a comparison. The MTT assay, commonly used as a measure of proliferation, is a measure of metabolic activity rather than proliferation as it relies on reduction of the MTT dye to formazan by NADPH dependent enzymes. As metabolic activity is increased during cell proliferation the MTT acts as an indirect measurement, therefore comparisons with the ^{3}H-thymidine assay must bear this in mind.

As with the ^{3}H-thymidine assay every cell line increased their metabolic activity in a concentration dependent manner with FBS (Fig. 6.2.5-3). The cell lines derived from both mild and moderate asthmatic patients, with the exception of htHB94, displayed an increase in metabolic activity following 10% FBS stimulation compared to the healthy derived line htHB96 however only the line htHB97 was significant ($p<0.001$). There was no clear correlation apart from this between the result and disease severity from which the cells were derived as both cell lines from severe asthmatics displayed significantly greater metabolic activity compared to one moderate line (htHB94) but significantly less than the other (htHB97).



6.2.5-3 Immortalised ASM cells were grown, serum starved for 72 hours and stimulated with varying concentrations of FBS for 48 hours. The cells are then incubated for 4 hours with 12mM MTT then a SDS-HCl solvent is added and incubated for a further 4 hours, plates are read at 574nm. Cell lines were either derived from healthy patients (white bar), mild asthmatics (light grey) or moderate asthmatics (dark grey). Bars represent mean ± SEM, n=12, analysed by one way-ANOVA and Bonferroni's multi comparison post test.

6.3 Discussion

Unfortunately, it is clear that interpreting the results outlined here and drawing more meaningful insights requires the same experiments to be performed in parallel on the native versions of each cell line. Without that comparison it cannot be deciphered whether the responses observed are due to the immortalisation

process or if they are typical of the patient they were derived from. Attempts were made to grow the control cells of each line for comparative parallel testing however they were of a high passage upon importation and as well as looking morphologically different, the cells did not grow to passage easily. The immortalisation is being carried out again on several new healthy and moderately asthmatic derived cell lines at an earlier passage while control cells are kept in the UK. Upon importation of the newly immortalised lines the characterisation will be far more accurate and useful. The data outlined here will serve as a "characterisation of the characterising process".

6.3.1 Staining

Each line was first stained with typical ASM markers to ensure they still expressed some of the defining proteins and they all came up positive. It has been previously published that native ASM cells decrease both α and γ-isoactin expression after the third passage (Panettieri *et al.*, 1989). Staining was repeated again for all of the markers, including α-actin, ten passages on from immortalisation. Although the immunocytochemistry was not quantified there appeared to be no reduction in sm-α-actin present indicating they are not subject to the same passage dependent phenotypic drift.

6.3.2 SERCA2 expression

A reduction in SERCA2 protein expression in asthmatics was put forward as "fundamental to many features of the exaggerated ASM function" (Mahn *et al.*, 2009) with the paper showing a clear negative correlation between expression and disease severity. An alteration in calcium handling in asthmatic cells was also postulated by Trian et al. (2007) as an explanation to increased proliferation and mitochondrial biogenesis observed.

In the cell lines tested here htHB95 derived from a healthy patient had markedly higher levels of SERCA2 compared to both moderate asthmatic lines htHB100 and htHB107, fitting with the published hypothesis. Beyond that there was no such correlation as both mild asthmatic lines exhibited very high SERCA2 expression. As western immunoblotting is only a semi-quantitative measure and is very dependent upon the quality of antibodies being used a functional measure of SERCA2 expression was employed.

6.3.3 Calcium transients

The calcium ion content of the sarcoplasmic reticulum depends upon the net balance between the rate of uptake by SERCA2 and the rate of basal leak from the store. Comparisons can therefore be made between the peak $[Ca^{2+}]_i$ response following SR dumping and the calcium content of the SR and thus indirectly gauge SERCA2 activity. A more direct measurement can be inferred by then calculating the rate of $[Ca^{2+}]_i$

falling back to basal levels following the agonist stimulation as the majority of calcium is pumped back into the SR and not out of the cell. This is best demonstrated in Brody's disease where SERCA2 expression is not affected only the function is which results in an increase of the half-life of the agonist response by three times compared to healthy muscle cells (Benders *et al.*, 1994).

The peak calcium responses following bradykinin stimulation only partially matched the SERCA2 protein expression data. Thus either indicating an uncoupling of expression and function or potentially highlighting the limitations to this assumption. The relationships that did match the expression data was that between the two lines derived from mild asthmatics and the two from moderate asthmatics. Both mild asthmatic lines had higher SERCA2 expression and higher peak responses to calcium. The decay rate back to baseline which is a more direct measure of SERCA2 function (Mahn *et al.*, 2009) (but omits the other variables of buffering to the mitochondria and pumping out of the cell) however did not match up with these results. In both cases the lines derived from moderate asthmatics had a faster decay rate indicating greater SERCA2 function which reached significance between htHB97 and htHB100 ($p<0.05$).

The healthy cell line htHB96 had quite a low relative expression of SERCA2 but the peak response to bradykinin and rate of decay were both considerably higher than that of the moderate asthmatic derived line htHB107 and slightly greater than htHB100 which is in line with the original hypothesis. Neither difference reached significance and in the case of htHB107 there is a distinct possibility of a type 2 error. The n value for this line was only 3 for the peak response and 2 for the decay rate owing to cells detaching from cover slips and erroneous results being generated. Increasing the repetitions of the test and hence increasing the power of the stats through reduction of variation may uncover a potential difference that has been missed.

6.3.4 Eotaxin-1 secretion

ASM cells derived from asthmatic patients have been shown to have both an enhanced synthetic and proliferative phenotype in culture (Wright *et al.*, 2012c). Eotaxin has been shown to be increased in the sputum of asthmatics and positively correlated eosinophilic airway inflammation (Yamada *et al.*, 2000). A range of mediators have been shown to influence its production; both the T_H2 cytokines IL-4 and IL-13 can induce eotaxin-1 expression in ASM and although TGF-β has no effect alone, when combined with the other two, the response is enhanced (Zuyderduyn *et al.*, 2004). TNF-α shows a similar effect by enhancing both IL-4 and IL-13 mediated production (Moore *et al.*, 2002; Odaka *et al.*, 2007). As shown in Chapter 3 both TGF-β and TNF-α modulate calcium homeostasis, furthermore reducing SERCA2

expression enhances eotaxin-1 release (Mahn *et al.*, 2009). This indicates that the cytokine storm in asthma may alter calcium handling, leading to various enhanced synthetic phenotypes such as eotaxin-1 production. Importantly, to address the aim of this chapter, eotaxin-1 production has been positively correlated with asthma disease severity making it a good index for the characterisation (Mahn *et al.*, 2009; Pepe *et al.*, 2005).

The correlation between SERCA2 expression and eotaxin-1 production was relatively poor in contrast to (Mahn *et al.*, 2009) however the results alone did match their eotaxin-1 data more closely. Both moderate asthmatic derived lines showed greater eotaxin-1 release compared to the healthy derived lines going against the causative relationship between it and SERCA2 expression but not necessarily a dysregulation of calcium homeostasis as a whole in the cell.

6.3.5 ASM proliferation

Contrary to well published data a negative correlation between DNA synthesis as asthma severity was observed in most of the cell lines with the exception of htHB95 (healthy) and htHB107 (moderate asthmatic). Although not fitting with their previously published population means the relationship between SERCA2 expression and DNA synthesis was maintained in htHB96 and htHB94. The healthy derived line htHB96 displayed low SERCA2 expression and high proliferation with the mild asthmatic line htHB94 showing the inverse. The MTT assay is often used as a parameter for cellular proliferation or viability as it measures NADPH dependent metabolic activity. The results obtained show increased activity with higher concentrations of FBS akin to the ^{3}H-thymidine assay but little similarities between the comparative cell line responses. A general trend towards higher metabolic activity was observed in the asthmatic cell lines compared to the healthy line however it wasn't severity dependent. The increased metabolic activity displayed in the asthmatic cell lines which isn't reflected in higher proliferation could be in part due to the elevated synthetic phenotype as observed with the eotaxin-1 production.

The two cell lines derived from mild asthmatic patients showed the least correlation to previously published results in regards to the parameters tested. As mentioned in the introduction, asthma is a heterogeneous disease and patients meeting only the criteria of a mild phenotype by our selection process may not display as many of the "typical" asthmatic phenotypes. The selection criteria for mild asthmatics was a predicted FEV_1 score of approximately 90% and this is taken at a screening visit up to a month before the biopsy is taken. The very nature of asthma manifests itself with variable lung function so many

of the patients screened into this group may not differ much from the healthy group or the moderate group depending upon their symptoms on the screening visit.

6.3.6 Conclusion

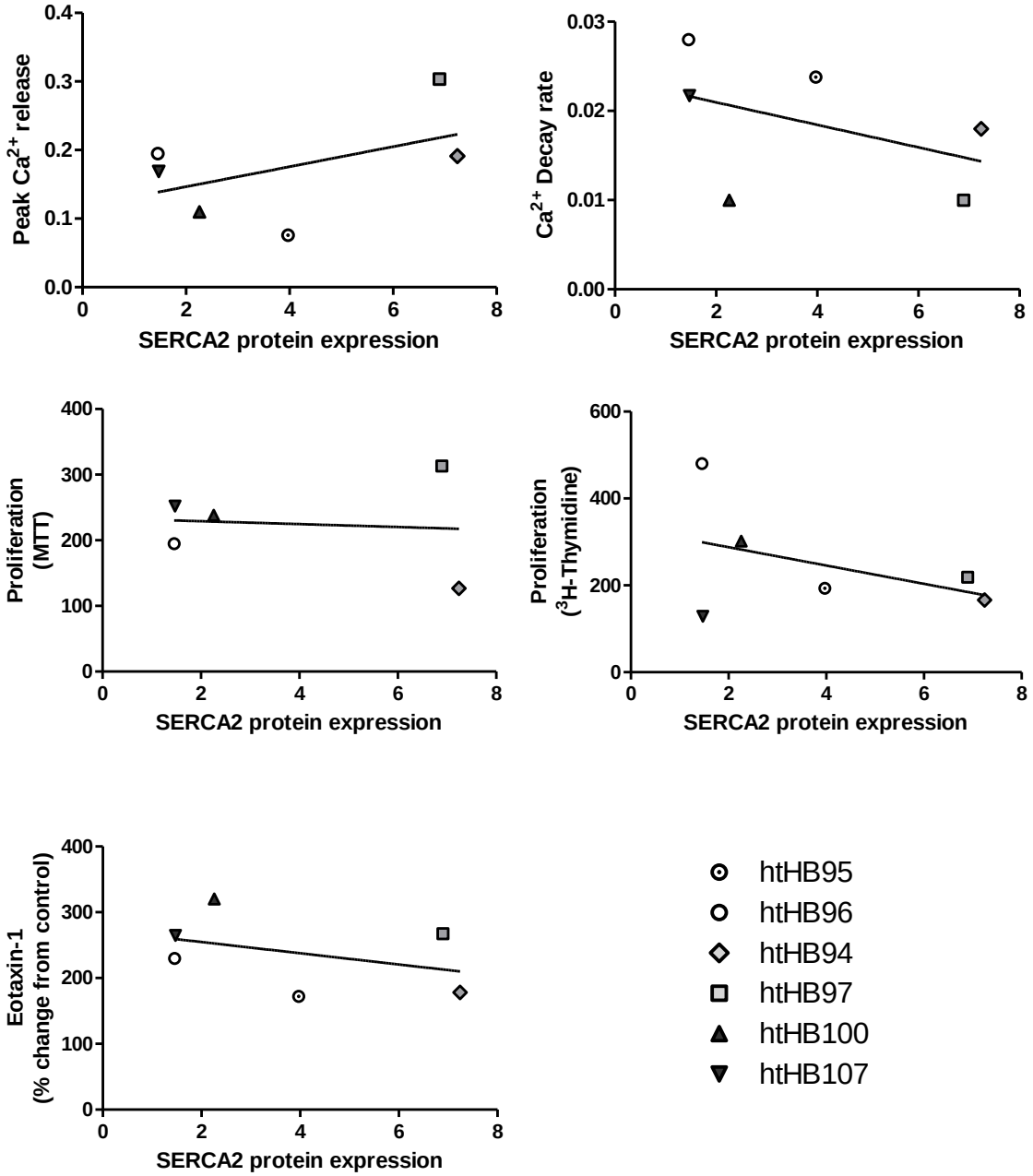
The data from the chapter can be approximately summarised in the table below to whether the data from each cell line fits with previously published reports:

	htHB95 (Healthy)	htHB96 (Healthy)	htHB94 (Mild)	htHB97 (Mild)	htHB100 (Mod)	htHB107 (Mod)
Staining	Yes	Yes	Yes	Yes	Yes	Yes
SERCA2 expression	Yes	No	No	No	Yes	Yes
Peak Ca^{2+}	No	Yes	No	No	No	Yes
Ca^{2+} recovery	Yes	Yes	Yes	Yes	No	No
Eotaxin-1	Yes	Yes	No	Yes	≈Yes	Yes
DNA synthesis	Yes	No	No	Yes	No	Yes
MTT assay	-	Yes	No	No	Yes	Yes

6.3.7 Correlations with SERCA2 expression

Fig. 6.3.7-1 below shows the data re-arranged to compare each parameter against SERCA2 protein expression as the underlying hypothesis states that this factor is the "driving force" for the other asthmatic phenotypes. The strongest correlation is with the peak calcium release data and SERCA2 expression with an r^2 of 0.24, the correlation is positive in fitting with the theory that higher SERCA2 expression leads to greater SR filling. The rate of calcium decay however is negatively correlated with SERCA2 protein expression which is contradictory to the hypothesis that SERCA2 expression heavily dictates the recovery rate. It is fitting with the disease state of each line as the healthy derived cells had faster rates of recovery therefore the quality of the western blot may be called into question or the regulation of SERCA2 activity.

Proliferation as measured by tritiated thymidine incorporation and eotaxin-1 release following IL-13 stimulation were both negatively correlated with SERCA2 expression as expected (r^2=0.19 and 0.16 respectively). The severity of asthma does not fit well with either factor so in these circumstances they do not serve as a strong model for the disease.



6.3.7-1 Each parameter tested plotted against SERCA2 protein expression on the x-axis to gauge the correlation with the initial hypothesis of reduced SERCA2 expression leading to enhanced asthmatic phenotype. Each point represents the mean of an individual cell line, healthy derived cells (clear circles), mild asthmatic (light grey quadrilaterals) and moderate asthmatics (dark grey triangles).

As mentioned, such simple conclusions cannot be made with confidence between the cell lines characterised as there is no fixed control to act as the benchmark. Without the non-immortalised versions of each line to compare against it is impossible to know which has been adversely affected by the immortalisation process. A comparison back to the population means using the raw data from (Mahn *et al.*, 2009) was attempted to gain further insight but not enough was available to draw further conclusions.

Several groups have been using immortalised ASM derived in the same manner as ours to generate important insights within the field (Dekkers *et al.*, 2010; Redhu *et al.*, 2009). However, the proper characterisation has not been carried out on these lines to discover what phenotypes have remained and which have been altered. To fully validate the work this needs to be achieved and it will add extra weight to the findings to show the key aspects still match native cells.

In conclusion, it appears as though the cell line htHB107 has maintained most of the characteristics expected of a cell line derived from a moderate asthmatic while htHB95 and 96 (derived from healthy patients) have maintained the majority of their disease-specific markers. The mild asthmatic derived cell lines htHB97 and htHB100 appeared to be the least characteristic but it is unknown whether this is due to the immortalisation process. The experiments outlined here could prove to be very useful in phenotype screening of newly immortalised lines to provide a robust research tool. It may prove hard to generate immortalised ASM lines showing every hallmark of asthma published therefore additional parameters could be added with the aim to develop disease specific sub-types.

Chapter 7 Final Discussion

Recent evidence shows that hospital admissions and mortality associated with asthma in the UK have reached a plateau and are showing signs of decline in recent years despite a relatively stable prevalence in the population (Anderson *et al.*, 2007). The paper postulates that pharmacological improvements in β_2-AR agonists and corticosteroid drugs may have played a role in this. Nevertheless there are still patient cohorts who do not respond well to current therapy and thus form the driving rationale to further understand the pathophysiology of the disease to generate new, novel targets.

Calcium homeostasis is undoubtedly altered in both healthy derived primary cultures of ASM treated with asthma associated cytokines and in naive asthmatic derived primary cultures. We have shown, with others, that varying expression levels of key calcium handling proteins in both scenarios are likely to underpin these changes. The work here has furthered the field by showing that a disruption of calcium handling proteins can enhance an asthmatic phenotype *in vivo* in terms of AHR and inflammation, specifically neutrophilia. We have also started to tease out the role TRPC6 may play in the altered ASM phenotype exhibited in asthma and the complex relationship it holds with TRPC3 and calcium entry. The data presented here is in accordance with White *et al*., 2006 where they proposed a switch from ROCE to SOCE upon TNF-α stimulation. We confirmed an increase in TRPC3 expression but also showed that TRPC6 decreases which fits with the current hypothesis. The exact relationship between ROCE, SOCE, TRPC3/6 and SERCA expression is a complicated affair. Evidence suggests that the SR, plasma membrane, cytosol and nucleus have a very dynamic relationship and that changes in calcium ion concentration can be directed in specific spatial and temporal patterns to encode complex signalling messages.

Measuring the function of SERCA2 in this thesis was performed in an indirect manner by measuring the exponential constant as calcium returned to baseline levels after stimulation. Due to technical limitations these measurement were acquired at room temperature and not 37°C which may lead to a deviation from the true *in vivo* contribution of it removing calcium from the cytosol (Mackiewicz *et al.*, 2006). The paper shows in cardiac myocytes that the contribution of NCX increases from 24°C to 37°C at the expense of SERCA in relaxation, therefore changes in function measured *in vitro* may not be as pronounced *in vivo*. Nevertheless the murine study outlined here shows that a reduction in SERCA2 expression to a similar level observed in cultured ASM derived from asthmatic patients *does* result in an enhanced asthmatic phenotype.

The scope of this thesis is limited in terms of its ability to extrapolate the changes in TRPC function to either synthetic or contractile ASM phenotypes which are altered in asthma. This can form the basis of future work to determine the relevance and impact of these findings on the pathophysiology of asthma. Furthermore, the *in vitro* data solely focuses on ASM cells in monoculture and the influence of their *in vivo* surrounding is missing. For example, the ECM can interact greatly with ASM and can modify its phenotype such as increasing proliferation as well as the compliance of the airways through integrin tethering (Hirst *et al.*, 2000; Johnson *et al.*, 2004b). As demonstrated with the *in vivo* SERCA2 data, not all of the characteristics were translated from Mahn *et al*., 2009 to the findings in Chapter 4 such as enhanced eotaxin-1 production. However a greater sensitivity for an increase in total lung resistance and decline in dynamic compliance was observed, a finding only made possible by studying the lung functioning as a whole organ.

From the data gathered in this thesis coupled with previously published work the key messages are: The expression of several calcium handling proteins are altered in asthma however the mechanisms behind them may differ. SERCA2 protein expression is reduced and maintained in asthmatic ASM cell culture for several passages hinting towards genetic or epigenetic mechanisms (Mahn *et al.*, 2009). Although it has previously been shown to also be reduced by inflammatory cytokines (Ojo, 2011; Sathish *et al.*, 2009) the evidence put forward here lies in contrast with that. The discrepancy at the very least sheds doubt on the findings. Conversely TRPC3 and TRPC6 are not altered in asthmatic derived primary ASM cultures compared to healthy but drastically and differentially changed in the presence of inflammatory cytokines. Therefore future work looking to model "asthmatic ASM" in culture might be improved by using cells derived from asthmatics in the presence of exogenously applied cytokines in order to achieve the full spectrum of alterations that are probably present *in vivo*. Secondly, that reducing SERCA2 *in vivo* enhances inflammation and AHR in a murine model of the disease. Whether the reduction is a cause or consequence of these characteristics in asthma is yet to be determined. Finally, that the increased morbidity observed with chronic β_2-AR agonist treatment is not a consequence of cAMP mediated decrease in SERCA2 expression leading to calcium handling disruption in ASM as previously hypothesised. It may possibly be the result of the non-canonical β-arrestin pathway disrupting calcium homeostasis but there is currently insufficient evidence to substantiate this claim.

The possibility of therapeutically targeting changes in $[Ca^{2+}]_i$ is attractive as it is a common secondary messenger for many of the phenotypes altered in asthma (Mahn *et al.*, 2010). The proteins controlling it however are very widely expressed and therefore will potentially have on-target side effect issues.

Therefore in order for this potential to be realised the fine intricacies of the signalling pathways must be unravelled to discover aspects which are specific for the ASM.

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